"Polymeric immunoglobulin receptor expression in the human sinonasal epithelium in chronic upper airway diseases : relation to eosinophilicinflammation and epithelial differentiation"

Hupin, Cloé

Abstract

Cette thèse s'intéresse aux mécanismes de défense de la muqueuse nasosinusienne au cours des principales maladies chroniques des voies respiratoires supérieures, à savoir la rhinosinusite chronique avec et sans polypes, ainsi que la rhinite allergique, par comparaison avec les maladies chroniques des voies respiratoires inférieures comme l'asthme et la BPCO. La question principale de ce travail est de savoir si les modifications épithéliales observées au préalable dans les maladies chroniques des voies aériennes inférieures (asthme et BPCO) se retrouvent également au niveau voies aériennes supérieures. Le système de l'IgA sécrétoire y est exploré au travers de son récepteur principal, le récepteur aux immunoglobulines polymériques (pIgR), dont la production et l'expression s'avèrent être diminuées dans les maladies liées à l'inflammation éosinophilique. Dans un deuxième temps, c'est l'épithélium nasosinusien lui-même qui est investigu&#65533;...

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Polymeric immunoglobulin receptor expression in the human sinonasal epithelium in chronic upper airway diseases: relation to eosinophilic inflammation and epithelial differentiation

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Abbreviations

AR: allergic rhinitis

BCs: basal cells

BM: basement membrane

CCs: ciliated cells

COPD: chronic obstructive pulmonary disease

CRS: chronic rhinosinusitis

CRSsNP: chronic rhinosinusitis without nasal polyps

CRSwNP: chronic rhinosinusitis with nasal polyps

ECP: eosinophil cationic protein

Hmw CK: high molecular weight cytokeratins

Ig: immunoglobulin

IL: interleukin

pIgR: polymeric Ig receptor

RT-qPCR: real time quantitative polymerase chain reaction

SA : Staphylococcus aureus

SAEB : Staphylococcus aureus enterotoxin B

S-IgA: secretory IgA

SC: secretory component

Summary

Chronic inflammatory diseases of the upper airways represent an important health burden, with major impacts on medical costs and public health[1, 2]. These inflammatory disorders are associated with abnormal responses to environmental factors and involve interactions between multiple factors. Despite active research in the field, fundamental mechanisms underlying the pathobiology of these disorders remain elusive.

Allergic rhinitis is clinically defined as a symptomatic disorder of the nose induced after allergen exposure by an IgE-mediated inflammation and considered as a classic Th2-mediated disease[3]. 17 to 29% of the European population suffers from allergic rhinitis, and prevalence as incidence are still increasing[1,4]. Chronic rhinosinusitis (CRS) seems to mirror the increasing frequency of allergic rhinitis, with an overall prevalence estimated around 11% in Europe[5, 6]. CRS is defined as an inflammatory disorder of the upper airways, which undergo distinct structural and functional changes of the sinonasal mucosa. At one time considered as a single disease entity, CRS is now commonly subdivided into 2 subtypes with differing pathophysiology, clinical expression and responses to treatment: CRS without nasal polyps (CRSsNP) and CRS with nasal polyps (CRSwNP)[2]. A variety of inflammatory mediators, including cytokines and chemokines, as well as adhesion molecules and matrix metalloproteinases, are upregulated in both subgroups of CRS, as is remodelling[7]. The tissue inflammatory response in CRSsNP is known to be neutrophilic with a Th1 skewing and high levels of IFN-gamma, whereas CRSwNP inflammatory responses are characterized by eosinophilia with a tendency toward Th2 polarization, high local IgE concentrations, IL-4, IL-5 and IL-13 expression[8].

Several specialized epithelial cells line the upper airways, playing a role both as mediators and regulators of immune and inflammatory response. The respiratory epithelium is continuously in contact with inflammatory and physical environmental stimuli, and provides frontline innate defence mechanisms, through a mechanical barrier function (mucociliary clearance) and the secretion of protective proteins[9]. Indeed, under physiological conditions, microorganisms are eliminated from the airways without involvement of the adaptive immune system. An impaired epithelial immune barrier function could thus be one of the causative mechanisms in CRS, compromising the pathogen-host interaction, making the sinonasal mucosa more susceptible to antigenic exposure and thereby leading to chronic inflammation[10, 11]. Indeed, these protective functions have been shown to be diminished in CRSwNP, allowing microbial colonization by Staph Aureus within the nose and sinus cavities[12].

A large spectrum of other alterations involving histology, T-cell patterns, remodeling parameters (eg, TGF-b), eicosanoid and IgE production, microorganisms, and epithelial barrier malfunctions has been reported to describe the pathogenesis of CRS[11].

Evidence of epithelial dysfunction in CRS has been described at different levels. First, the physical epithelial barrier is compromised or disarrayed. This defect may facilitate the passage of allergens and other foreign agents into the airway tissue, leading to immune activation and thereby further stimulating the inflammatory process. This has been shown in vitro[13] as in vivo, in human[14] and animal models[15].

It has also been shown that the chemical epithelial barrier was altered, with a modified number of mucus-producing goblet cells [16, 17], altered antioxidant

levels[18] and changes in the airway surface liquid which contains various proteins and peptides that provide an antimicrobial shield; these include lysozyme, lactoferrin, secretory leukocyte proteinase inhibitor, elafin, secretory phospholipase A2, anionic peptides, cathelicidin and β-defensins[19].

Finally, the immunological epithelial barrier has been shown to be modified, with various cytokines that are either upregulated or downregulated. This will be discussed in details in the first chapter.

CRS results thus, at least in part, from a dysfunction of the nasal epithelium in its ability to orchestrate appropriate and regulated immune responses to foreign matter.[20] As an implied corollary, defects in the mechanical barrier and innate immune response would result in appropriate recruitment and persistence of adaptive immune responses with development of the clinical symptoms characteristic of the disease.[20]

A major frontline defense mechanism, common to the different mucosal surfaces, consists of secretory immunoglobulin A (s-IgA). IgA is the most abundant Ig isotype in nasal secretions, and produced by mucosal plasma cells, after what it is transported across mucosal epithelial cells. This transport is mediated by a transmembrane glycoprotein called the polymeric immunoglobulin receptor (pIgR). After transcytosis, IgA is released at the apical surface after proteolytic cleavage of the pIgR extracellular domain known as secretory component (SC).

The first aim of this work was to investigate whether the active transport of IgA was affected in chronic sinonasal pathologies, through a reduced

expression of pIgR in the nasal mucosa, as described previously in the bronchial epithelium of severe COPD patients[21]. Indeed, we observed a reduction of the pIgR expression in CRS, but unexpectedly in the CRSwNP phenotype, as well as in AR (and not in CRSsNP). This defect was associated with decreased SC and IgA antibodies to bacterial antigens in nasal secretions of CRSwNP patients, in parallel to subepithelial accumulation of IgA, and related to eosinophilic, Th2-related inflammation.

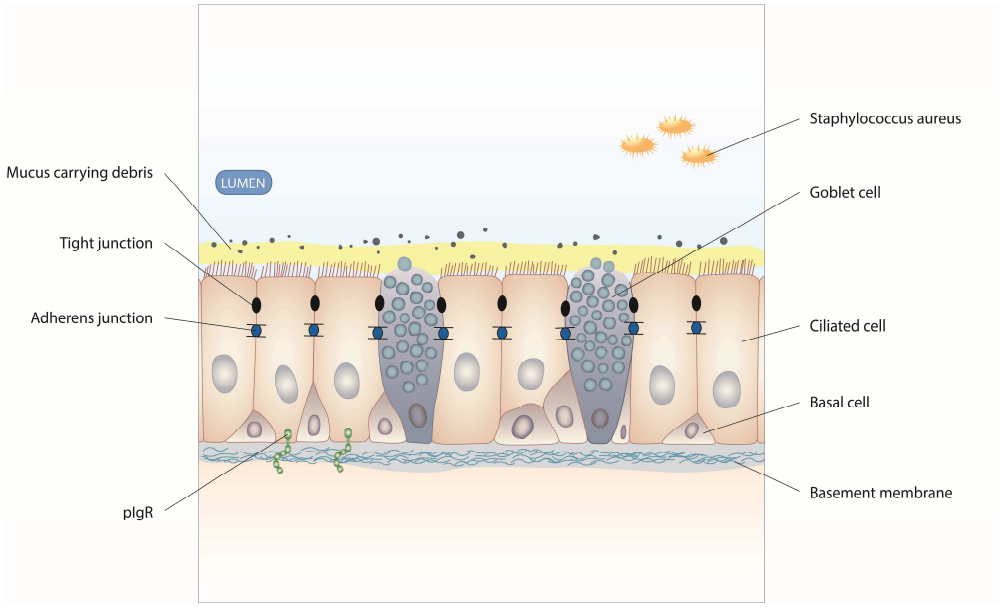

Figure 1 : Normal upper airways epithelium

In addition to inflammation, it is increasingly evident that structural changes are present in chronic upper and lower airway diseases, as referred to as remodelling[22, 23]. Tissue remodelling in upper airways includes increased extracellular matrix deposition and subepithelial basement membrane

thickening[23]. A particular mechanism of remodeling and epithelial differentiation is represented by epithelial-to-mesenchymal transition (EMT), consisting of the loss of the epithelial phenotype by epithelial cells and acquisition of a mesenchymal phenotype[24]. EMT has been identified in the lower airways from patients with COPD[24] and asthma[25]. Whereas features of EMT have been reported in chronic upper airway diseases[13, 26-28], its existence in the upper airways has not been demonstrated yet.

Since our team showed recently that pIgR downregulation is closely related in COPD to altered airway epithelial cell differentiation through TGF-β activation, thereby resulting in impaired lung IgA immunity in these patients[29], we wondered whether pIgR downregulation in CRSwNP could be due to de-differentiation of the upper airway epithelium.

The second aim of this work was thus to assess whether epithelial dedifferentiation via EMT occurs in CRSwNP. We show that the sinonasal epithelium undergoes dedifferentiation towards a more mesenchymal phenotype in CRS, and that this occurs independently of CRS phenotype (CRSwNP and CRSsNP) and of lineage specification into ciliated and goblet cells.

A third aim was to assess whether IgA responses could be modulated upon therapy, such as antibiotics, corticosteroids, anti-IgE or anti-IL5 biotherapies. Therefore, we assessed different subclasses of IgA antibodies in CRSwNP patients treated by omalizumab or mepolizumab, as well as doxycycline and methyprednisolone, as compared to placebo. Only very modest differences were observed in these data, reinforcing the need for further studies looking at effects of therapies on local IgA production.

Finally, the main question of this thesis is to know if the epithelial modifications observed previously in chronic diseases of the lung, namely asthma and COPD, could also be found at the upper level of the airways, during chronic sinonasal diseases.

Thus this thesis explores the pathobiology of the upper airway epithelium in chronic rhinosinusitis and shows that it undergoes important changes including pIgR downregulation and dedifferentiation through mesenchymal transition, also further highlighting common features of epithelial reprogramming in chronic disorders of both upper and lower airways.

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CHAPTER 1: INTRODUCTION

Immune defence mechanisms: comparing upper and lower airways in chronic airway diseases

Immune defence mechanisms: comparing upper and lower airways in chronic airway diseases

Cloé Hupin, Philippe Rombaux, Marylène Lecocq, Birgit Weynand, Yves Sibille, Charles Pilette, *Immunology, Endocrine and Metabolic Agents – Medicinal Chemistry* 2010; 10 (3):123-141

ABSTRACT

Several epidemiological, pathophysiologic and clinical data demonstrate the interrelationship between upper and lower airways, and common features between inflammatory pathways and disorders of the nasal and bronchial mucosa have been emphasized both in the clinic (ARIA guidelines[1]) and in basic science, and reflected by numerous reviews on this topic. In contrast, detailed comparative analysis of basic defence mechanisms in upper versus lower airways and its clinical relevance is lacking.

It is becoming increasingly clear that airway epithelium plays a key role in driving key initiating steps of immune defence (either protective or deleterious) against inhaled antigens and particles. Epithelial cells respond to changes in the external environment by secreting a large array of antimicrobial host defence molecules, cytokines and chemokines following exposure to and activation by pathogen molecular patterns, and link in concert with dendritic cells innate to antigen-specific adaptive immunity. However, the type of epithelial response to 'pathogens' and danger signals may differ between upper and lower airways, as well as according to genetic background.

This review provides an updated, extensive and comparative review of immune defence mechanisms of the respiratory tract, in upper *versus* lower airways, including most relevant changes observed during chronic airway diseases and how these may translate into different clinical features.

INTRODUCTION

The airway epithelial cells work as a complex physical barrier but also play a crucial role in initiating and perpetuating airway host defence mechanisms. Mechanical defences include the deposition on the nasal and oropharyngeal surfaces and elimination through cough, sneezing and mucociliary clearance[2]. If particles or microorganisms reach the alveolar space despite these mechanical defences, alveolar macrophages insure their removal. Moreover, epithelial cells mediate innate host defence through production of antimicrobial molecules including antimicrobial peptides, pro-inflammatory cytokines, growth factors, enzymes and chemokines[3]. The production of these substances is initiated in response to activation via pattern recognition receptors (PRR)[3]. When the innate immune functions fail, the epithelium also induces the transition from innate immunity to adaptive immunity. Epithelial cells are capable of directing dendritic cells towards a response to antigen exposure[4] and contribute to the local recruitment of phagocytes and antigen-specific T- and B-cells of the adaptive immune system, to eliminate the infection[5].

An increasing number of studies defend the concept of United Airways, suggesting that nasal and bronchial mucosa should be seen as a continuum, in particular in inflammatory reactions where inflammation in one part of the airway influences its counterpart at distance.

Although much of the immunopathology is shared between the upper and lower airways, there are marked differences in the pattern of inflammation in the respiratory tract, with different inflammatory cells, mediators, consequences, and responses to therapy.

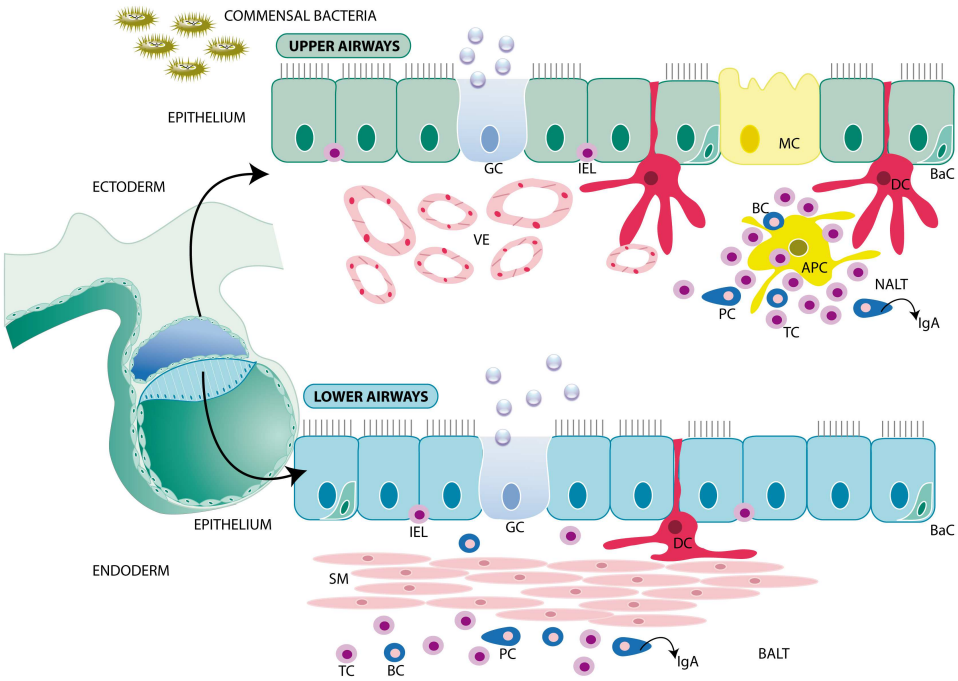

Figure 1. Differences between upper and lower airways. Whereas the epithelium shares several features, the nose originates from the ectoderm while lungs from endoderm. The immune system includes in upper airways the NALT, while immune cells do not display constitutively such organisation in the lower airways. Upper airways are physiologically in close contact with commensal microbes, while distal airways are supposed to be sterile. The structure of upper airways includes a large supply of subepithelial capillaries, arterial systems and venous cavernous sinusoids, while smooth muscle is only present in the bronchi. Finally, upper and lower airways differ by their physical function: sneezing, air warming, and deposition of large particles are properties of upper airways while cough and deposition of smaller particles occur in lower airways; both levels sharing mucociliary clearance as common physical defence.

GC, goblet cell; IEL, intraepithelial lymphocyte; MC, mast cell; VE, vessels; SC, stem cell; DC, dendritic cell; BC, B cell; TC, T cell; APC, antigen presenting cell; PC, plasma cell; NALT, nasal associated lymphoid tissue; BALT, bronchial associated lymphoid tissue; BaC, basal cell

1. CLINICAL DEFINITION AND IMMUNOPATHOLOGY OF CHRONIC AIRWAY DISEASES

Airway inflammation is observed in a wide range of respiratory conditions affecting upper and/or lower airways, which are caused or influenced by several components including genetic background (e.g., α1-antiproteinase deficiency, ADAM-33 polymorphism) and environmental exposure (allergens, microbes, irritants such as cigarette smoke). Some are closely linked to atopy (allergic rhinitis and asthma) and others to cigarette smoke (COPD).

a. Allergic rhinitis

Allergic rhinitis (AR) is an IgE-mediated inflammatory disorder due to abnormal responses of the nasal mucosa to inhaled allergens, characterized by a mixed inflammatory infiltrate made up of eosinophils, T cells (of the Th2 phenotype), mast cells and basophils[6]. Symptoms include sneezing, anterior or posterior aqueous rhinorrhea, nasal itching and nasal obstruction, on exposure to sensitizing antigens. AR affects patients of all ages, all countries, all ethnic groups and all socioeconomic conditions[1], with a prevalence rate ranging from 17 to 29% in Europe[7]. Ocular symptoms have been estimated to be present in 40–60% of the allergic population[8]. Ocular symptoms may present in allergic rhinitis, and less frequently may be associated in asthmatic patients[8].

b. Chronic rhinosinusitis

Chronic rhinosinusitis (CRS) is characterized by persistent symptoms (more than 12 weeks) related to inflammation in the paranasal sinuses, defined

clinically and on CT and/or endoscopic evaluation. Depending on the presence of polyps on endoscopy, CRS can be divided into CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP). CRSwNP and CRSsNP share most of their clinical features –with smell loss characteristic of NP, but these two entities may be distinguished by their respective T-cell cytokine profiles. CRSwNP displays Th2 polarization with IL-5 expression, eosinophilic infiltrate, and high local IgE concentrations, while CRSsNP is considered as Th1-biased, with high levels of IFN-γ[9].

Table 1 summarizes the recent definition proposed by the EAACI[10].

Inflammation of the nose and paranasal sinuses characterized by:

- 2 or more symptoms, one of which should be either nasal obstruction or discharge :
    - Nasal blockage, congestion
    - Nasal discharge or postnasal drip
    - Facial pain or pressure
    - Reduction or loss of smell
- And either
    - Endoscopic signs
        - Polyps and/or
        - Mucopurulent discharge from middle meatus and/or
        - Oedema/mucosal obstruction primarily in middle meatus
- And/or
    - CT changes:
        - Mucosal changes within ostiomeatal complex and/or sinuses

Table 1 Clinical definition of rhinosinusitis including nasal polyposis[10]

c. Asthma

Asthma is a chronic inflammatory condition of the lower airways defined by largely reversible airflow obstruction, airway hyperresponsiveness and mucus hypersecretion, leading to episodic respiratory symptoms; these include wheezing, cough, breathlessness and chest tightness. As in AR, Th2 cells orchestrate the inflammatory response in asthma through the release of IL-4, IL-13, IL-5 and IL-9[11]. Asthma is characterized by an inflammatory process in which the immune response to allergens is mediated by mast cells, CD4+ Th2 cells, eosinophils, dendritic cells, and IgE-secreting B cells. Following exposure to proteolytic allergens, the airway epithelium condition through cytokines called “alarmins” (TSLP, IL-33, IL-25) dendritic cells to drive in the regional lymph nodes naive CD4+ T cells to differentiate into Th2 cells. In turn, Th2 cytokines drive cardinal features of asthma: IL-4 and IL-13 promote B cells class-switch recombination to IgE synthesis; IL-5 induces eosinophil chemotaxis, maturation and survival ; IL-9 promotes mast cell development; and both IL-9 and IL-13 regulate mucus secretion and airway hyperresponsiveness, in asthma and in AR [12]. Both antigen-driven and nonspecific mechanisms are then able to lead to respiratory symptoms such as sneezing, watery rhinorrhea, wheezing and chest tightness.

Asthma and AR are frequently associated. Between 20 and 50% of patients with AR have asthma, and 30 to 90% of patients with asthma have concomitant AR[13;14].

d. COPD

COPD is defined on lung function testing by poorly reversible airway obstruction. The disease is often associating damage to proximal bronchial airways (including epithelial mucous metaplasia) and to distal airways and airspaces (alveolar destruction, as referred to emphysema). Cigarette smoking is by far the most important risk factor in the development of COPD, followed by occupation and air pollution[15]. According to some studies, COPD develops in around 25% (15 to 50% according to different studies) of heavy smokers[16-18].

More recently, inflammation has also been recognized as a hallmark in COPD[19]. A distinguishing feature of COPD is the local accumulation of macrophages, neutrophils and CD8+ T-cells as well as a type 1 (Th1/Tc1) cytokine profile[11], while Th2-related inflammation with eosinophilic infiltration could be seen during some exacerbations[20] and in COPD patients with hyperresponsiveness improving on corticosteroids.

Inhaled irritants, such as cigarette smoke, activate epithelial cells and macrophages to release multiple cytokines, including IL-8, tumour necrosis factor (TNF)-α, IFN-γ or TGF-β, which stimulate fibroblast proliferation, resulting in fibrosis in the small airways[11;21].

2. CELLULAR INFILTRATION

Immune cells infiltrating the airways during such diseases could be divided into two distinct groups according to their presume roles; *effector leukocytes* include neutrophils, macrophages, eosinophils, mast cells, basophils and B-

lymphocytes, whereas *regulatory leukocytes* are represented by T-lymphocytes and dendritic cells.

Neutrophils have the capacity to induce oxidative damage and proteolysis through the release of preformed mediators including lysozyme, lactoferrine, cathelicidins, defensins, serine proteases (neutrophil elastase, proteinase-3 and cathepsin G), as well as matrix metalloproteinase (MMP)-8 and 9[5;22].

Basophils and mast cells can also release preformed inflammatory mediators such as histamine, prostaglandin D2, cysteinyl leukotrienes, and neutral proteases. These mediators cause sensory neural stimulation and plasma exudation from blood vessels, which the patient experiences as itching, sneezing, nasal discharge, and congestion as well as lower respiratory symptoms[23]. These cells can also elaborate newly synthesized factors, such as leukotrienes and IL-4, contributing notably in allergy to late-phase responses to allergen exposure. Eosinophils have a capacity to release tissue-toxic granule proteins, leukotrienes and numerous cytokines and are predominant infiltrating cells in allergic airway disease (AR and asthma) and in CRSwNP[24]. Macrophages represent resident phagocytes of lower airways, that may be activated by cigarette smoke or pathogens to secrete several inflammatory mediators and proteins such as oxygen-derived radicals, MMP-2, -9 and -12 (macrophage elastase) as well as cathepsins[22].

Activated mast cells, eosinophils, and basophils infiltrate the airways of asthmatics as a result of an excessive Th2 response, while in COPD infiltration of neutrophils, macrophages and CD8+/Tc1 lymphocytes is observed[11]. Neutrophilic infiltration in both the airway lumen and tissues

has been shown to correlate with disease severity[25]. In contrast to asthma, eosinophils are not increased in COPD, except in case of asthmatic features such as hyperresponsiveness or corticoid reversibility or during some exacerbation[26].

The predominant inflammatory cells in AR consist of eosinophils, basophils, lymphocytes, macrophages and plasma cells[27]. Increased numbers of T cells have also been reported in CRS, CRSsNP typically showing a predominant Th1-cytokine profile and infiltration by neutrophils and macrophages[28]. Conversely, CRSwNP displays a Th2-skewed eosinophilic inflammation dominated by eosinophils and mast cells[28]. Nasal polyps include dense concentrations of eosinophils in a stroma that may be variably dense or loosely oedematous[29].

	AR	**CRSsNP**	**CRSwNP**	**COPD**	**Asthma**
Effector cells	Eosinophils Basophils	Neutrophils Lymphocytes Macrophages	Eosinophils Lymphocytes	Neutrophils Macrophages Lymphocytes	Eosinophils Mast cells Lymphocytes
CD4 profile	Th2	Th1	Th2	Th1	Th2

Table 2: Inflammatory and immune cell infiltration in upper and lower airway diseases

Regulatory cells (Treg cells) are T cells able to suppress effector T cells, both from Th1 or Th2, or Th17 phenotype. Treg-mediated suppression involves cell-cell contact (e.g. through CTLA4) and/or secretion of inhibitory cytokines such as IL-10 or TGF-β. Inhibition of the development of allergy by Tregs may occur through several mechanisms, including suppression of effector T cells or direct effects on inflammatory cells such

as eosinophils, mast cells / basophils, or B cells. Also, they favour isotype switching from IgE to IgG4[30].

3. IMMUNE DEFENCE MECHANISMS

The immune defence mechanisms of the nasal mucosa consist of a physical and chemical barrier to inhaled microbes, particles and antigens. The respiratory epithelium is actively involved in immune responses, innate immune recognition relying on a limited number of germline-encoded *pathogen related receptors* (PRRs) such as Toll-like receptors (TLR) and nucleotide binding oligomerization domains[31]. These receptors recognize a variety of conserved molecules, commonly present on viruses and bacteria named *pathogen associated molecular patterns* (PAMPs), including lipopolysaccharide (LPS), lipoteichoic acid (LTA), mannans, glycans or bacterial/viral nucleic acids (double or single-stranded DNA and RNA) and which signal downstream to intracellular cascades that result in the gene transcription of antimicrobial and inflammatory cytokines[32]. Other functions of PRRs include opsonization, phagocytosis, and induction of apoptosis[32]. Signals provided by TLR are essential to induce dendritic cell maturation that subsequently allows priming of naive T cells during primary immune responses to a newly recognised antigen[33]. Through induction of costimulatory molecules and cytokines, the innate immune system also primes adaptive immune responses[34]. PRRs are expressed on most cells of the immune system such as macrophages, dendritic cells, B-cells as well as on respiratory epithelial cells, but may also be resident in intracellular compartments or secreted as soluble molecules into the bloodstream and tissue fluids [5].

a. The respiratory epithelium

The airway epithelium constitutes the interface between the host and the external environment and is thereby constantly exposed to potentially infectious agents such as bacteria, viruses, fungi and parasites, as well as potentially harmful soluble molecules from the environment. It produces a large array of molecules and mediators (Table 3 and 4), whereas it could also play a suppressive role on immune responses to harmless antigens. Thus, epithelial cells, beyond their role as a physical barrier, are active players during innate and adaptive immunity.

Sinonasal and bronchial mucosa are similar histologically, both being characterized by a ciliated pseudostratified columnar epithelium resting on a basement membrane and covered with a bilayer of surface fluid, with a superficial gel or mucous layer and a layer of periciliary fluid interposed between the mucous layer and the epithelium. In the normal maxillary sinus, more than 90% of the mucosal surface area is covered with cilia[35]. Further down to proximal bronchi, distal (membranous) bronchioles are lined by an columnar epithelium becoming more cuboidal[36]. Concomitantly to disappearance of cartilage and submucosal glands, distal (or 'peripheral', 'small') airways are characterized by the emergence of Clara secretory cells[36]. At least eight morphologically distinct epithelial cell types have been observed in the bronchial airways, although based on ultrastructural, functional and biochemical criteria these may be classified into three main categories: basal, ciliated and secretory[37]. In large airways (20 to 2^5 branches in humans), the most represented cell types are ciliated, secretory, basal cells, and undifferentiated columnar cells. In distal airways (2^6 to 2^{23}

branches), similar cell types are observed, with increased proportion of ciliated cells and a shift of secretory to Clara cells. After 2^{23} branches, the airway epithelium (in respiratory bronchioles) merges with the alveolar epithelium, with mostly type I and type II cells[36].

Mucus-secreting goblet cells are present all along the respiratory tract. In the upper airway mucosa, their density varies from one sinus to another, with a maximal density of 10.000 cells/mm² in the maxillary sinus[8]. In the bronchi, goblet cells represent 5 to 15% of the columnar cell population, their proportion decreasing in more distal airways.

The airway epithelium is a source of numerous mediators in allergic inflammation, but might also play a key primary role during sensitization. Functional dysregulation of the so-called epithelial-mesenchymal trophic unit has been suggested in asthma[39], favouring Th2-type allergic inflammation through abnormal repair processes and production of proinflammatory factors, but it remains controversial whether this occurs also in the nose.

Defence mechanisms of the respiratory tract		
	Humoral	**Cellular**
Mechanical	Mucociliary clearance	Ciliated epithelium
Adaptive Immunity (Specific)	Immunoglobulins	Lymphocytes (T and B cells)
Innate Immunity (Aspecific)	Glycoproteins • Mucins • Lysozyme • Lactoferrine • pIgR • Surfactant proteins • Clara protein • sPLA2 Anti-inflammatory and antibiotics • Neutral endopeptidase • Annexins • Defencins • Cathelicidins Anti-proteases • SLPI • Alpha-1-antitrypsin Eicosanoids • Leucotriene B4 • Prostaglandins • Lipoxin Cytokines	Granulocytes Macrophages

Table 3: Defence mechanisms of the respiratory tract (Adapted from Aubier et al. Traité de Pneumologie 2ème edition) pIgR, polymeric immunoglobulin receptor; sPLA2, secretory phospholipase A2; SLPI, secretory leukocyte proteinase inhibitor

b. Remodelling

Remodelling is defined as a process leading to transient or permanent changes in tissue architecture, which involves changes in tissue structures (basement membrane and interstitial stroma) that result from insults that are not adequately balanced by appropriate repair mechanisms. There is now very strong evidence that structural components are playing a driving role in inflammatory airway disease, along with immunological mechanisms *per se*. Functional changes in the function of epithelium and mesenchymal tissues, referred morphologically to as "remodelling", may lead to vicious circles of impaired frontline defence mechanisms (mucociliary clearance, epithelial proteins including defensins, secretory-IgA) and exaggerated immune cell recruitment. Recent studies show that impairment of innate protective mechanisms of the airway epithelium, such as production of TGF-α[40] and IFN-β[41] is part of abnormal epithelial repair responses to viral infections in asthma. Most of this evidence of such implication of structural tissues –including epithelial cells and (myo)fibroblasts– has thus been obtained in asthma, and whether this extends to rhinitis remains uncertain[42]. Some studies suggest similar mechanisms in allergic rhinitis in terms of epithelial shedding[43] and myofibroblast activation[44], but this was not confirmed by others[45]. Increased vascular permeability[46] and angiogenesis[47] has been observed in allergic rhinitis. Also there is evidence of persistent epithelial activation in perennial allergic rhinitis, such as ICAM-1 expression[48]. If it appears that tissue changes occur in the upper airways to a lower extent than in lower airways, it is also clear that remodelling processes are engaged in allergic rhinitis, including increased expression of

TGF-β[49] and VEGF[50], even if final consequences on tissue structure might not be discerned as easily as in lower airways in asthma. Other factors that could contribute to chronicity of airway inflammation include superantigens from commensal Staphylococcus aureus[51] and exposure to a range of environmental stimuli such as viruses, tobacco smoke and ambient air pollution.

A typical change observed in many chronic airway disorders consists of hyperplasia and metaplasia in mucus-secreting cells, particularly observed in the bronchi[52]. During inflammatory responses in the airways, resolution occurs through apoptosis of inflammatory leukocytes that have been recruited and activated locally, while repair of collateral damage to neighbouring tissue – in particular to the surface epithelium – includes regeneration from epithelial basal cells that can differentiate into a specialized cell type. These basal cells serve as progenitor niches within proximal airways - from the nose to large bronchi[52], whereas in distal airways studies indicate that Clara cell (and Clara cell variants) or basal cells could play this role[53].

c. Mucociliary clearance

Mucociliary clearance is considered the first line of defence against bacteria deposited in the airways, acting as a physical barrier[54]. The epithelial lining fluid covering the upper and lower airways is a twofold layer consisting of a superficial gel or mucous layer and an underlying, watery fluid that surrounds the cilia on the apical surface of the ciliated cells, called airway surface liquid (ASL)[55]. The mucus is produced by surface goblet cells and by submucosal glands. The gel-like properties of the mucus depend

primarily on their content of high molecular weight glycoproteins known as mucins, which behave as a tangled three-dimensional polymer network. These macromolecules immobilize inhaled microbes and particles into the mucus and/or destroy them[54]. Surrounding macrophages can engulf and destroy bacteria but also release cytokines that will attract neutrophils and other cells[56]. Effective mucociliary clearance depends on the viscoelastic properties of mucus and requires coordinated ciliary activity. This basic physiological properties of mucus and ciliary function are impaired in most chronic disorders such as chronic sinusitis[57] and COPD[58], at least in part as a result of secondary ciliary dyskinesia.

In addition to physical properties of mucus, ASL contains multiple proteins and peptides that provide an antimicrobial shield; these include lysozyme, lactoferrin, secretory leukocyte proteinase inhibitor (SLPI), elafin, secretory phospholipase A2 (sPLA2), anionic peptides, cathelicidin (LL-37) and β-defensins[56]. These anti-microbial factors have many roles during innate immune responses, including broad spectrum antimicrobial activity, the ability to act as chemokines as well as to induce chemokine production leading to recruitment of leukocytes to the site of infection, the promotion of epithelial wound repair and the ability to modulate adaptive immunity[59]. Although several antimicrobial peptides are constitutively expressed, their expression and secretion can be triggered through several pathways, including proinflammatory cytokines as well as bacteria or PAMPs[60]. Some of these factors, especially lysozyme, lactoferrin and SLPI, interact to enhance their activity[61].

d. Defence (glyco)proteins

i. Mucins

The major protein component in the mucus is a family of highly glycosylated, viscoelastic, disulfide-bonded glycoproteins named gel-forming mucins. Mucins are characterized by a large molecular weight (2–20 x 10^5Da), high carbohydrate content reflecting a large number of *O*-glycans, and numerous tandem repeats in the protein backbone[62]. They are secreted in vesicles derived from the Golgi apparatus, stored in the cytoplasm of goblet cells and released by exocytosis at their apical surface in response to mucin secretagogues[38]. Of the currently known 20 mucin genes that encode protein backbone of mucins, 16 have been identified in the airways[38]. The epithelial mucins are extremely hydrophobic and are associated with various macromolecules, the quality and quantity of which may also affect the physicochemical properties of the mucus.

A wide variety of stimuli, including allergens, bacteria, mechanical injury, cigarette smoke and cytokines can induce mucus secretion via epidermal growth factor receptor (EGFR) expression and activation, causing goblet-cell metaplasia from Clara cells by a process referred to as cell transdifferentiation[63]. Since airway inflammation is usually accompanied by mucus hypersecretion, any condition causing inflammation is likely to stimulate mucin release in the airways, either directly or indirectly[64]. Accordingly, many chronic inflammatory diseases of the lower respiratory tract are associated with mucus hypersecretion, which contributes to airway obstruction in patients with asthma, COPD, or cystic fibrosis[62]. Mucus hypersecretion is also associated with AR[65] and CRS[66], either with or

without nasal polyps, characterized by increased numbers of goblet cells within the surface epithelium[63] and/or increased numbers of submucosal glands[67].

ii. Lysozyme and lactoferrin

Lysozyme and lactoferrin are the most abundant antimicrobial factors in nasal secretions[68]. Lysozyme is a 14kDa polypeptide contained within both phagocytic and secretory granules of neutrophils, whereas it is also produced by monocytes, macrophages, and epithelial cells[5]. Lysozyme can destruct the cell wall from bacteria by cleaving glycosidic bonds of *N*-acetylmuramic acid, and induces cell lysis. It is also toxic for various fungi but has no specific impact on antiviral defence. Increased production of lysozyme has been observed in the nasal mucosa of patients with chronic rhinosinusitis[69] and in BAL fluid of patients with COPD[70]. Conversely, a significant decrease in lysozyme levels was reported in patients with allergic rhinitis and CRS compared to patients with allergic rhinitis alone or controls[71]. Lysozyme C precursor has also been shown to be down-regulated in CRS patients when compared to normal subjects[72].

Lactoferrin, as lysozyme, is a major component of secretory granules of neutrophils and is found in high concentration in several biological fluids. Its structure is very similar to the serum iron transporter transferrine. Although chelation of iron represents its key biological function, this 80kDa glycoprotein is also very prone to bind other macromolecules such as IgA, secretory component and lysozyme[73]. Almost all microorganisms need iron for growth, therefore by sequestering iron and making it inaccessible to invading organisms lactoferrin exerts a bacteriostatic effect on Gram

positive and Gram negative bacteria and yeasts[74;75]. Other activities include anti-inflammatory activity and the regulation of cell cycle and differentiation[76]. Lactoferrin secretion has been shown to be 2-fold higher in bronchial than in nasal mucosa[77], and enhanced concentrations have been found in BAL fluid from patients with COPD[70] or asthma[78]. In contrast, recent studies reported a reduction in lactoferrin expression in the nasal mucosa of CRS patients, at both mRNA and protein levels[79].

iii. pIgR and IgA

S-IgA, the most abundant Ig isotype in mucosal secretions, is involved in both innate and adaptive immunity as it can be produced either with or without defined antigen specificity. High affinity IgA antibodies play a critical role in protective adaptive immunity, while 'low affinity' IgA antibodies (sometimes referred as to 'natural antibodies') represent a high capacity innate, frontline defence system[80]. B cells in upper airways are initially stimulated in organized mucosa-associated lymphoid tissue, known as nasal associated lymphoid tissue (NALT), which includes the tonsils and adenoid. The existence of bronchial associated lymphoid tissue (BALT) remains elusive in homeostasis but does emerge in the lower airways from pathology, particularly in COPD[81] From these inductive sites, memory B cells migrate to secretory effector sites where they differentiate into terminal Ig-producing plasma cells[82]. Monomeric IgA (mIgA) is constituted by two non-specific light chains covalently associated with two specific heavy chains and has a molecular weight of 160kDa[2]. IgA is synthetized by mucosal B cells in the mucosal lamina propria as dimers, with two IgA monomers joined by the joining (J) chain in a "tail-to-tail" conformation,

whereas larger polymers (trimers, tetramers, and even pentamers) can also be found in low amounts.

Polymeric IgA must be translocated from the subepithelial area into mucosal secretions, across the epithelium. A specific receptor for polymeric Igs called polymeric Ig receptor (pIgR), expressed at the basolateral pole of epithelial cells, is assuming this active transport. IgA is transcytosed by the pIgR up to the apical surface where a proteolytic cleavage releases the main part of the pIgR extracellular domain known as secretory component (SC), which helps protecting the molecule from proteolysis[2]. The pIgR is expressed in bronchial and nasal mucosae, as well as in the digestive and urogenital tracts, and S-IgA in respiratory secretions may exert protective anti-microbial activities by scavenging pathogens and antigens. As pIgR transcellular routing also occurs without IgA, free SC is released which is also able to contribute to pathogen scavenging. pIgR expression and function is upregulated through multiple signaling pathways initiated by PAMPs and pro-inflammatory cytokines such as IFN-γ, IL-4, TNF-α, and IL-1[4;83]. However, reduced pIgR expression has been observed in the airways of severe COPD patients, and this impairment correlated with airflow obstruction and with neutrophil infiltration of submucosal glands[2]. A significant increase of IgA has been shown in tissue homogenates, but not in serum, of CRSwNP patients, when compared to CSRsNP patients and controls, suggesting a local production[84].

iv. Surfactant proteins

Surfactant is a lipoprotein complex composed of 90% phospholipids and 10% proteins, designated as SP-A, SP-B, SP-C, and SP-D[85;86]. SP-A and SP-D are hydrophilic glycoproteins, members of the collectin family, which exhibit

antimicrobial and interact with a variety of bacterial, viral and fungal pathogens[86]. In addition, SP-A and SP-D can interact with dendritic cells and modulate subsequent T-cell responses, optimize leukocyte function and chemotaxis, and affect subsequent cytokine/chemokine profiles and activate complement[87]. Conversely, SP-B and SP-C are hydrophobic proteins which increase the rate which surfactants spread over the surface[85]. Surfactant proteins have long been considered lung-specific but recent studies showed that SP-A[88], SP-B[89] and SP-D[86] - but not SP-C - are also expressed in the nasal mucosa.

v. Clara cell protein

Clara cell protein (CC10, also referred to as CC16 or uteroglobin) is a 15.8-kDa homodimeric protein secreted in large amount in distal airways (bronchioles) where Clara cells are localized[90], whereas gene expression can be detected along the tracheobronchial tree. CC10 is a steroid-inducible protein with immunomodulatory (mainly through inhibition of phospholipase A2) and antiproteinase properties[91] which helps to protect the lung during local inflammatory responses[92]. The secretion of CC10 is upregulated by cytokines such as TNF-α[93] or IFN-γ[94]. The serum concentrations of CC10 are decreased in subjects with asthma[91], whereas results in COPD have been more contradictory. Decrease in CC10 has been reported in the serum from COPD in one study (78), but not in another[91]. Decreased CC10 content has also been observed within proximal and distal lower airways from severe COPD patients and cystic fibrosis patients[95] as well as in smokers without COPD[2]. In the BAL fluid of asthmatic patients, CC10 has been shown to be 10-fold lower than in healthy volunteers[96]. There is increasing evidence that acute exposures to respiratory irritants could cause transient increases in serum CC10 levels[90;97]. Whereas Clara cells in

humans are restricted to terminal bronchioles, CC10 protein has also been found in nasal secretions[98] and could be involved in the pathogenesis of upper airways diseases. Recently, it was shown that CC10 production is reduced in CRS with or without NP[97] as well as in allergic rhinitis[99].

vi. sPLA2

Phospholipase A2 (PLA2) enzymes are a family of esterases which play a major role in the generation of two inflammatory lipid mediators: eicosanoids and platelet-activating factor (PAF). Among them, ten secretory PLA2 (sPLA2) have been identified and described as structurally related, disulfide-rich, low-molecular mass enzymes with strict Ca2+ dependence[100]. sPLA2 are usually expressed and released by granulocytes (mast cells, basophils, eosinophils) and Th2 cells[101]. They are involved in hydrolysis of outer cell membrane phospholipids and in antibacterial defence through maturation, recruitment, and activation of inflammatory cells[100]. sPLA2s also induce degranulation and production of cytokines and chemokines from inflammatory cells, such as monocytes/macrophages and eosinophils[101].

sPLA2s are released in the airways of patients with allergic asthma and rhinitis[101], and Lindbom et al. found a large number of PLA2 types in nasal and paranasal fluids and mucosal tissues from control and allergic rhinitis patients[102]. In addition, Touqui et al. showed that sPLA2 activity increases in nasal lavage fluid from allergic patients after allergen provocation[103]. Recently, Liu et al. have showed that the expression of some members of group II subfamily of sPLA2s (sPLA2-IIA, IID, IIE) is upregulated in CRSsNP[100]. When comparing polyp tissue and non-polyp sinusal tissue, they found that polyps displayed significantly lower sPLA2-IIA mRNA and

higher expression of sPLA2-IIE mRNA, than specimens from CRSsNP patients[100]. Similarly, in lower airways, bronchial instillation of sPLA2 can induce bronchoconstriction and tissue damage[104], and elevated levels of sPLA2 are found in BAL[105] and sputum[106] from asthmatics.

e. Anti-inflammatory and antimicrobial proteins/peptides

i. Neutral endopeptidase

Neutral endopeptidase (NEP) is a highly selective glycoprotein, regulating the activity of neuropeptides released in the respiratory mucosa[107]. In the airways, NEP represents the major enzyme which reduces cellular responses to neuropeptides. NEP secretion is regulated by the glandular, cholinergic system[108]. Functions of respiratory epithelia and inflammatory cells are influenced, at least in part, by biologically active agents released from both sensory and efferent autonomic nerves, and neurogenic inflammation has been shown to be different in upper and lower airways. The nasal mucosa is especially densely innervated by sensory nerves which, when stimulated, activate protective reflexes such as sneezing, rhinorrhea and nasal congestion, in order to assure first line defence against pathogens and particles[109]. On the other hand, lower airway neurogenic inflammation is characterized by plasma protein extravasation, airway smooth muscle contraction and increased secretion of mucus[110]. Reduced peptidase activities in serum or BAL fluid are found in healthy smokers when compared to non-smokers[111], suggesting that smoking may affect *per se* NEP-mediated control of neurogenic inflammatory responses.

ii. Annexins

Annexin A1, also known as lipocortin-1 or calpactin II, is a calcium-dependent glucocorticoid-inducible protein. Annexin A1 has been implicated in various cellular processes such as neutrophil migration, apoptosis, intracellular signal transduction, membrane-cytoskeletal linkage, regulation of cell growth and differentiation[112-114]. Annexin-1 also inhibits the activity of cytosolic PLA2 (cPLA2). Given that cPLA2 plays a key role in the release of arachidonic acid for the production of eicosanoid inflammatory mediators, the inhibition of this enzyme by annexin-1 is thought to contribute to its anti-inflammatory activity[112]. Annexin A1 was found to be constitutively expressed in normal nasal mucosa and is detected in bronchoalveolar lavage fluid[114].

Enhanced annexin A1 mRNA and protein expression was reported during chronic inflammation of the nasal mucosa[114]. In contrast, Lindahl et al. showed a significant reduction of lipocortin-1 in subjects with rhinitis, when compared to healthy subjects[96], as well as in nasal epithelial cells from CF patients[115]. It has been suggested that expression of annexin A1 in the nasal epithelium could be related to the cellular differentiation status rather than chronic inflammation[113]. In a mouse asthma model, allergen-induced oxidative stress results in proteolysis of annexin A1 and subsequently up-regulation of cPLA2 activity and LT production[116]. In humans, higher levels of annexin A1 are present in the BAL fluid of asthmatic patients and smokers, when compared with healthy volunteers[117].

iii. Defensins

Defensins are endogenous small highly cationic peptides found at epithelial surfaces, that can be divided into two main subgroups, namely α- and β-defensins which differ in their distribution and connection of six cysteine residues[118]. While α-defensins are produced by neutrophils and intestinal Paneth cells, β-defensins are mainly expressed by epithelial cells[119]. Both subclasses possess antimicrobial activity against Gram-positive and Gram-negative bacteria, enveloped viruses, mycobacteria and fungi[5;120]. This activity relates to their ability to disrupt the membranes of a wide range of organisms, causing permeability changes and cell death[120]. Defensins are chemotactic for T cells and therefore may be capable of activating T cell-dependent immune responses[121]. Beta-defensin genes are induced in the airways by bacteria and TLR agonists[122].

Recent studies reported overproduction of defensins in CRS patients either with[123] or without[124] nasal polyps, when compared to healthy volunteers, while low concentrations of β-defensins-4 have been observed in patients with AR[125]. In the lower airways, elevated defensins levels were also observed in sputum from patients with CF[126] while reduced defensin activity due to elevated salt concentration has been implicated in the pathogenesis of CF lung disease[127]. Upregulation of defensins 1 and 2 was also documented in BAL fluid of smokers with COPD compared to asymptomatic smokers[128], and recent studies reported associations of a polymorphism in the gene encoding human *β*-defensin-1 with asthma[129] and with COPD[130].

iv. Cathelicidins

Cathelicidins, like defensins, are produced by various cell types, including neutrophils, macrophages, dendritic cells, T cells and epithelial cells[131]. The only cathelicidin expressed in humans, LL-37, has been shown to have a broad-spectrum activity similar to that of defensins, against both Gram-positive and Gram-negative bacteria, as well as Candida albicans, and is able to neutralize LPS[132]. It mediates a wide range of biological responses: direct killing of microorganisms, chemoattraction of leukocytes, chemokine/cytokine release, lung epithelial cell proliferation, neutralisation of endotoxins such as LPS, stimulation of angiogenesis, as well as promotion of wound closure of the airway epithelium[133].

Recent studies showed that LL-37 mRNA was increased in nasal polyps[134] and in CRS patients[135] when compared to normal nasal mucosa. Similarly, increased concentration of LL-37 was detected in the sputum of COPD patients, whereas reduced levels were observed in asthma[136].

v. Anti-proteinases

SLPI is a 12-kD non-glycosylated tissue-specific inhibitor of serine proteases, produced by epithelial cells at mucosal surfaces. By inhibiting neutrophil elastase, cathepsin G and mast-cell chymase, it prevents damage to the ciliated epithelium during bacterial infections[137]. Recent studies indicate that SLPI also exhibits anti-inflammatory properties[138] and provides a weak but broad-spectrum antimicrobial activity, killing micro-organisms and limiting viral spreading[139]. Atopic patients have a lower concentration of SLPI in nasal secretions than healthy subjects, independently of antigen exposure[137], whereas a rise in SLPI is observed following antigen

challenge[137]. No data are reported regarding SLPI in CRS, while in lower airways a reduction of SLPI has been shown in COPD[140]. Its level in sputum of COPD patients varies inversely with infection[141] and the concentration of neutrophil elastase[142], and its activity is affected by oxidative stress[143]. Hollander et al. did not find significant differences in BAL fluid levels of SLPI between COPD and asthma patients[144].

vi. Lipid mediators (eicosanoids)

Arachidonic acid is the initial substrate for lipid-derived mediators, also called eicosanoids, representing plasma membrane phospholipid-derived polyunsaturated fatty acids. These mediators, which include leukotrienes (LTs), prostaglandins (PG) and lipoxins (LXs) are generated subsequently to the hydrolytic action of PLA2 on cellular phospholipids to release arachidonic acid, via the cyclooxygenase or lipoxygenase pathways[145]. Eicosanoids are produced by airway epithelial cells under baseline conditions or in response to various stimuli, actively perpetuating chronic inflammation[145]. Eicosanoids play an important role in the pathophysiology of airway diseases associated with inflammation, platelet aggregation and vasoconstriction/relaxation dysbalance.

Cysteinyl leukotrienes (CysLTs) are a family of pro-inflammatory lipid mediators synthesized by a variety of cells, including mast cells, eosinophils, basophils, and macrophages and to a lesser extent T cells and endothelial cells[146;147]. CysLTs have potent effects on vasodilation and leakage, bronchoconstriction, mucus secretion, collagen synthesis, leukocyte trafficking, epithelial proliferation, P-selectin increase, as well as production, adhesion, migration and survival of eosinophils[147;148]. In the

lung, CysLTs also augment growth factor-induced airway smooth muscle mitogenesis[149], in addition to their direct bronchoconstrictive capacity.

CysLT are increased in nasal secretions from patients with allergic rhinitis, following local antigen challenge and natural (seasonal) allergen exposure[148], and correlate with the intensity of the allergen provocation and the clinical response[150]. Increased concentrations of CysLT have also been observed in nasal secretions, nasal mucosa or polyp tissue from patients with CRSwNP[151] and their concentration correlated with disease severity[152] and eosinophilic inflammation[153]. Also CysLT levels are increased in the urine[154], BAL fluid[155] and sputum[156;157] of asthmatic patients; increased sputum levels correlating with eosinophils[158] and disease severity[156]. Such increase has also been reported in sputum of COPD patients[157].

Prostaglandins. The capacity of the epithelium for CysLT synthesis is inversely related to its ability to produce PGE2, which is the predominant eicosanoid product of the airway epithelium[149]. PGE2 is catalyzed by cyclooxygenase (COX) 2 in response to stimulation by many inflammatory cytokines such as platelet-derived growth factor (PDGF), IL-1β and TNF-α[159;160]. PGE2 is produced within the nasal and bronchial mucosa, but it remains unclear whether it can play a deleterious or a beneficial role in pathology. Several studies showed that PGE2 has a protective and anti-inflammatory role in asthma, with an inhibitory effects on eosinophils trafficking, release of inflammatory mediators, and allergen-induced airway responses[161]. Moreover, numerous studies have shown the ability of PGE2 to induce bronchodilation[162], whereas other prostanoids such as TXA2, PGD2 and PGF2a mediate varying degrees of bronchoconstriction[163]. PGE2

has also important roles in mitogenesis inhibition, collagen synthesis and mesenchymal cell chemotaxis. On the other hand, recent studies demonstrated PGE2 as a pro-inflammatory mediator that responds to inflammatory cytokine stimulation[159], enhances mucin gene expression[164] and eosinophil survival[165], and regulates Th17 development[166].

PGE2 also modulates the immune response by regulating macrophages, T and B lymphocytes leading to pro- and anti-inflammatory effects[167]. The existence of four PGE2 receptors, each with distinct signalling pathways, provides a plausible explanation for the apparent contradictory actions of this lipid mediator[168]. Several studies revealed increased expression of PGE2 in patients with asthma[165] and COPD[159;160] and PGE2 concentration were directly correlated with eosinophils in asthma[165] and with airflow limitation in COPD[159]. However, Kostikas suggested that the elevation of prostaglandin E2 in patients with asthma could mainly be attributed to smoking[169]. Indeed, deficient PGE2 production under proinflammatory conditions has been demonstrated in asthmatic airways[170].

On the other hand, PGE2 is significantly decreased in peripheral blood cells and in nasal polyp tissue as compared with normal nasal mucosa[152] and the PGE2 concentration could be inversely correlated to eosinophilic inflammation[152] and the radiological severity of sinusitis[171]. In patients with allergic rhinitis, lower local levels of PGE2 were reported, when compared with normal mucosa[172].

The reason for the apparent discrepancy of PGE2 levels between allergic rhinitis and asthma might relate to several factors among which are timing of sampling according to the kinetics of immune responses to antigen

exposure and methodological issues. Also, differences in receptor ligation and signalling in target cells may account for different effect of PGE2. Thus, PGE2 appears to regulate inflammation in a complex way, with subtle differences between patients according to primary pathology, as well as to airway level and smoking status.

Lipoxins. LXs are also generated by the metabolism of arachidonic acid, via enzymatic conversion by 12/15-lipoxygenase[152]. These molecules are the originally described endogenous lipid mediators of the resolution phase of inflammation. LX A4 is a short-acting, naturally occurring eicosanoid with potent anti-inflammatory actions, which can provide suppressive signals for leukocyte trafficking[152]. LXA4 is generated in asthmatic responses[173] and inhibits airway hyperreactivity and lower-airway inflammation[174]. Several studies showed decreased levels of LXs in the airways of severe asthmatic patients[157], while no data are reported in COPD. In the upper airways, nasal polyp tissue displays high LX production[175] and enhanced LX levels were shown in CRS, either with and without nasal polyps, compared with control subjects[152].

	AR	CRSsNP	CRSwNP	COPD	Asthma	Refs
Glycoproteins						
Mucins	Increased	Increased	Increased	Increased	Increased	62;63;65;66
Lysozyme	Unknown	Contradictory	Unknown	Increased	Increased	69-72
Lactoferrine	Unknown	Decreased	Unknown	Increased	Increased	70;78;176
CC10	Decreased	Decreased	Decreased	Decreased	Decreased	2;96;177
SPLA2	Increased	Increased	Contradictory	Unknown	Increased	100;105;106
Anti-inflammatory and antimicrobial peptides						
Annexins	Contradictory	Contradictory	Contradictory	Unknown	Contradictory	120;123;125;128
Defensins	Decreased	Increased	Increased	Increased	Unknown	120;123;125;128
Cathelicidins	Unknown	Increased	Increased	Increased	Decreased	134-136
Anti-proteinases						
SLPI	Decreased	Unknown	Unknown	Decreased	Decreased	22;137;140;144
Eicosanoids						
LT B4	Increased	Increased	Increased	Increased	Increased	148;151;155-157
PGs	Decreased	Decreased	Decreased	Increased/ Decreased	Increased	152;159;160;165;170-172
LXs	Unknown	Increased	Increased	Increased	Decreased	152;157;175

Table 4: Inflammatory mediators in upper and lower airway diseases

f. Cytokines

Cytokines play a critical role in orchestrating the immune and inflammatory responses, as well as structural changes of the respiratory tract, by recruiting, activating and promoting the survival of leukocytes[11] and by acting on structural cells. According to a large body of literature in mice and in humans, T-cell derived cytokines are now frequently used as 'biomarkers' to phenotype the immunopathological profile of immune-mediated diseases, including in the respiratory tract (Table 5). Besides the T-lymphokines, other cytokines play important roles in regulating immune responses to pathogens or to *intrinsic* factors underlying the disease.

T-cell-derived cytokines. Based on their pattern of cytokine secretion, CD4+ lymphocytes can be functionally divided into a Th1 or Th2 phenotype. Cytokines generally produced by one T cell subset inhibit the development of the other subset, leading to polarization of the immune system. Th1 cells, through the release of cytokines such as IFN-γ and TNF-α, activate macrophages and cytotoxic T lymphocytes (CD8+ CTL). In contrast, Th2 are involved in atopic diseases by secreting notably IL-4, IL-5, IL-9 and IL-13. These cytokines trigger humoral immunity (IgG and IgE secretion) and orchestrate the recruitment, proliferation and activation of basophils, mast cells and eosinophils[29]. Thus the presence of eosinophils in the airways is strongly linked to that of Th2 cells[178], which produce IL-5 as principal pro-eosinophilic cytokine. In the bronchi, asthma is associated with Th2 cell cytokine responses[179], while COPD is considered as a Th1-biased disease. A similar dichotomy between Th1-Th2 polarization is described in the sinonasal mucosa, with IFN-γ and TGF-β being

predominant in CRSsNP and IL-5 characterizing CRSwNP[180]. In AR, T-cell activation is also indicating a Th2 profile. However, in humans, allergen-stimulated T cells from atopics are also able to produce IFN-γ at baseline, and the Th1/Th2 ratio may increase following allergen stimulation[181]. Impaired production of both IL-10 and IL-12 has been observed in blood monocytes from atopic patients with asthma[182], suggesting a deficient capacity of these antigen presenting cells to drive Treg and Th1 immune responses to allergens in these subjects genetically susceptible to develop atopic diseases.

A third subset of effector T helper cells, called Th17 cells, are characterized by the production of various cytokines, including IL-17A, also called IL-17, IL-17F, IL-6, TNF-α, IL-21 and IL-22. Many of the inflammatory effects of Th17 cells are attributed to the expression of IL-17. IL-17 acts on airway epithelial cells, lung fibroblasts and other types of inflammatory cells to trigger the production of pro-inflammatory cytokines, chemokines and MMP, promotes the recruitments of neutrophil and macrophage, and leads to tissue inflammation[183].

An increase of IL-17 has been shown in serum[183;184] and bronchi[185] of asthmatic patients, and in the bronchi[186] of COPD patients, as well as in allergic rhinitis[187] and CRSwNP[185].

The helper T cell subsets have grown in numbers, with Tregs and Th17 enriching the Th1/Th2 families. More recently, a specific T cell subset secreting IL-9 (Th9) has been reported[188]. These Th9 cells are related to Th2 cells, but they have lower expression of other Th2 cytokines[189] Interestingly, TGF-β reprograms Th2 cells to lose their characteristic profile

and switch to IL-9 secretion or, in combination with IL-4, drives the differentiation of Th9 cells directly[189]. An increase in the expression of IL-9 mRNA has been shown in COPD patients[190], and IL-9 expression is increased in asthma[191]. It is also increased in CF, suggesting that IL-9 is a key mediator of mucus hypersecretion in various chronic airway diseases. In the nasal mucosa, IL-9 is upregulated during the pollen season and correlates with tissue eosinophils in patients with allergic rhinitis[192].

Regulatory T cells (Tregs) are T cells that are thought to suppress excessive (allergic) or misguided (autoreactive) immune responses that can be harmful to the host, in order to maintain peripheral tolerance. Different CD4+ Treg subsets have been identified, which include adaptive Treg cells and naturally occurring Treg cells characterized by the CD25 forkhead box P3 (FOXP3) phenotype.

The Treg cell response is characterized by abolished allergen-induced specific T-cell proliferation (as referred to as “anergy”) and suppressed Th1- and Th2-type cytokine secretion. The increased levels of IL-10 and TGF-β that are produced by Tregs, potently suppress IgE production, while simultaneously increasing production of non-inflammatory isotypes IgG4 and IgA, respectively. In addition, Tregs directly or indirectly suppress effector cells of allergic inflammation such as mast cells, basophils, and eosinophils[193]. A relative defect of peripheral and local CD4+CD25+ Treg function has been observed in allergy[194] and in allergic asthmatics[195;196], allowing Th2 cells to expand. An increased number of Treg cells in the BAL of COPD patients has been observed, but also in smokers lungs, so this increase could be associated to long-term cigarette smoke exposure[197].

Van Bruaene et al. showed a lower level of FOXP3 in CRSwNP, signing a decrease of Tregs, while no significative difference was noted in CRSsNP[198].

TSLP Thymic stromal lymphopoietin (TSLP) is an IL-7-like cytokine, initially identified in thymic stromal cells, but predominantly produced by epithelial cells and activated mast cells[199]. Through a potent activation of myeloid dendritic cells, TSLP can promote naïve CD4+ T cells to differentiate into Th2 cells producing IL-4, IL-5, and IL-13, while inhibiting the production of IL-10 and IFN-γ[200]. Increased epithelial TSLP expression can be elicited by exposure to viral or bacterial pathogens and/or by ligating TLRs[201], and further increases upon Th2 stimulation[202]. Overproduction of TSLP has been described in the epithelial layer of nasal mucosa of patients with AR[200], and Schleimer et al. found elevated TSLP mRNA levels in CRSwNP but without changes in TSLP protein[202]. High amounts of TSLP have also been found in the airways of asthmatic subjects[203] and 'asthmatic mice'[204], in line with the Th2 hypothesis. However, Ying et al. recently showed similar increases of TSLP in COPD patients[205].

BAFF B cell activating factor belonging to the TNF family (BAFF) is a member of the TNF ligand superfamily. BAFF is a key regulator of immunoglobulin class-switch recombination and B lymphocyte development, survival, proliferation and maturation[206]. BAFF is produced, by bronchial and nasal epithelial cells[206]. Levels of BAFF protein are significantly increased in BAL fluid after allergen challenge in allergic subjects[206]. An overproduction of BAFF in nasal polyps has also been observed in patients with CRSwNP[207].

	AR	CRSsNP	CRSwNP	Asthma	COPD	Refs
IL-4	Increased	Increased	Increased	Increased	Increased	22;97;208-212
IL-5	Increased	Increased	Increased	Increased	?	9;22;209;210;212
IL-9	Increased	?	?	Increased	Increased	190-192
IL-12	Decreased	Decreased/ Increased	?	Decreased/ Increased	Increased	182;213-216
IL-13	Increased	Increased	Increased/ No difference	Increased	Not increased	184;198;212;217-221
IL-17	Increased in severe AR	?	Increased	Increased	Increased	183-185;187
IL-18	Increased	?	?	Increased/ Decreased	Increased	184;222-224
IFN-γ	Decreased	Increased	Decreased	Decreased	Increased	28;184;198;208;225;226
IL-1β	Increased	Increased	Increased	Increased	Increased	28;208;227
IL-6	Increased	Increased	Increased	Increased	Increased	184;209;213;227-232
TNF-α	Increased	Increased	Increased	Increased	Increased	11;28;208;227;230;232-235
TSLP	Increased	?	Increased (mRNA)	Increased	Increased	200;202-205
GM-CSF	Increased	Increased	Increased	Increased	Increased	11;236-239
TGF-β	Increased/ Decreased	Increased	Decreased	Increased	Increased	28;198;208;240-246
IL-8/CXCL8	Increased	Increased	Increased	Increased	Increased	28;136;144;208;217;227;230;231;233
IL-10	Increased/ Decreased	?	?	Decreased/ Increased	Decreased	182;184;217;247;248

Table 5: Cytokines in upper and lower airway diseases

CONCLUSION

In conclusion, this report is reviewing the extensive data related on immune defence mechanisms operating in upper versus lower airways, as well as on their changes in chronic airway disorders. In particular, it is very likely that the airway epithelium plays a crucial role in key steps of chronic respiratory diseases, both during their development and natural history.

Many similar features can be found between upper and lower airway, e.g. patterns of inflammation in diseases such as type 2 (Th2) cell activation in CRSwNP and asthma and type 1 (Th1) in CRS and COPD, while proinflammatory cytokines such as TNF-α and IL-6 may amplify the inflammatory reaction and thereby determining disease severity.

However, several differences in immune and structural responses may be observed:

First, frontline defence molecules of innate immunity may vary between upper and lower chronic airway diseases; these include lactoferrine which is decreased in CRSsNP while increased in asthma and COPD (unknown in AR and CRSwNP) - differences have also been reported for lyzozyme, annexins, and SPLA2 (Table 4).

Second, mucosal associated lymphoid tissue is only present in upper airways under normal conditions, maybe in relation to the abundant presence of commensal bacteria that may provide signals to drive such immune orgnanisation and function; in contrast BALT seems to develop only upon pathological immune responses to foreign or endogeneous molecules (e.g. COPD, rheumatoid bronchiolitis).

Third, activation pathways leading to synthesis of growth factors such as TGF-β, EGFR ligands and/or VEGF, and/or the tissue reactivity and activation through their receptors, may vary between the nose and bronchi – and may be differently modulated by each other (e.g. TGF-β - EGFR crosstalk)[249] - and could explain that structural remodelling during chronic/repeated epithelial damage predominantly occurs in lower airways.

Fourth, clinical translation of inflammatory responses may vary between upper and lower airway levels according to the intrinsic function and/or structure of the airway, e.g. mucosal blockade due to vasodilation in the nose versus bronchospasm in the bronchi.

Further investigations are needed to better understand how the genetic background may imprint both immune and structural reactivity to inhaled materials in upper and lower airways, and to underlie e.g. increased susceptibility to inhaled allergens and/or biotoxics notably at the level of dendritic cells and the epithelium, and how this integrates the structural remodelling of the respiratory mucosa that is characteristic of chronic airway diseases.

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CHAPTER 2: OBJECTIVES OF THE THESIS

Objectives of the thesis

The specific objectives of the thesis were:

1. To assess whether the expression of pIgR/SC is reduced in CRSsNP, as compared to CRSwNP, AR and controls, and whether this change induces reduced IgA levels in mucosal secretions. The two subclasses of IgA were assessed, as well as specific IgA antibodies to relevant bacterial antigens such as S. pneumoniae and S. aureus. These changes were correlated to eosinophilic and T cell-driven inflammatory features.

2. To assess whether the upper airway epithelium undergoes dedifferetiation in CRSwNP as a feature underlying pIgR downregulation, and whether this is associated with epithelial-to-mesenchymal-transition and changes in lineage specification into goblet or ciliated cells. These findings were also correlated to subepithelial airway fibrosis and disease severity.

3. To assess whether secretory IgA responses in CRSwNP could be restored by therapies such as anti-IgE and anti-IL5 biotherapies or other anti-inflammatory treatments (corticosteroids, doxycycline). We assessed IgA antibodies in samples from CRSwNP patients included in placebo-controlled trials performed at the UZ Gent (Pr Ph Gevaert) for omalizumab, mepolizumab, doxycycline, and methylprednisolone, as compared to baseline and placebo.

CHAPTER 3: IS POLYMERIC IMMUNOGLOBULIN RECEPTOR-MEDIATED SECRETORY IMMUNOGLOBULIN A PRODUCTION IMPAIRED IN CHRONIC UPPER AIRWAY DISEASES?

Chapter 2: Is polymeric immunoglobulin receptor (pIgR)-mediated secretory immunoglobulin A production impaired in chronic upper airway diseases?

INTRODUCTION

The nasal mucosa is exposed to a large variety of inhaled microorganisms and antigens, and early recognition of antigen products in this region is critical for host defense.

As a protective mechanism, a major function of mucosal epithelial cells is the transport of dimeric immunoglobulin A (IgA) into external secretions. IgA is commonly recognized as the most prevalent antibody subclass at mucosal sites, with various functional attributes, both direct and indirect, that serve to prevent infective agents such as bacteria and viruses from breaching the mucosal epithelial barrier. High affinity IgA antibodies play a critical role in protective adaptive immunity by neutralizing microbial toxins and pathogens, while 'low affinity' IgA antibodies represent a high capacity innate, frontline defence system, by preventing commensal bacteria from breaching the mucosal surface[1].

Mucosal secretions contain mainly dimeric IgA. Plasma cells in the lamina propria secrete dimeric IgA (dIgA), which structurally consist of two IgA monomers joined with the J-chain in a "tail-to-tail" conformation.

To pass through the mucosal epithelial cells in order to enter the lumen, IgA requires a specific receptor for polymeric Igs called polymeric Ig receptor (pIgR), an integral membrane glycoprotein selectively expressed on the basolateral side of epithelial cells in the gut, bronchi and nasal mucosa.

Dimeric IgA is actively transported by the pIgR, which, thereby, plays an intrinsic role in protecting the respiratory tract from invading pathogens. After transcytosis, IgA is released at the apical surface after endoproteolytic cleavage of the pIgR extracellular domain or secretory component (SC) that bounds to dimeric IgA to form secretory IgA (s-IgA) and protects the molecule from proteolysis within the mucus[2,3]. SC displays itself, independently from IgA, some anti-infectious properties[1].

The specific humoral local defense is mostly mediated by s-IgA which is the most abundant Ig isotype in nasal secretions. S-IgA is involved in both innate and adaptive immunity and plays a crucial role in the first line of defense of the respiratory tract.[1] S-IgA is mainly produced as an "innate" Ig upon mucosal stimulation by microbial signals acting through Toll-like receptors on epithelial cells and B cells[4] while some IgA is produced as high-affinity antibody to particular antigens.

This transcytosis of the pIgR/IgA complex represents the most important transcellular routing in the body.

Several pathways may upregulate pIgR expression and/or transcytosis, including pro-inflammatory cytokines (IFN-γ, IL-4, TNF-α, IL-1), hormones and phorbol esters via various transcriptional and post-transcriptional mechanisms[5,6]. The expression of polymeric immunoglobulin receptor (pIgR) is also known to be increased in response to viral or bacterial infections, linking innate and adaptive immunity.

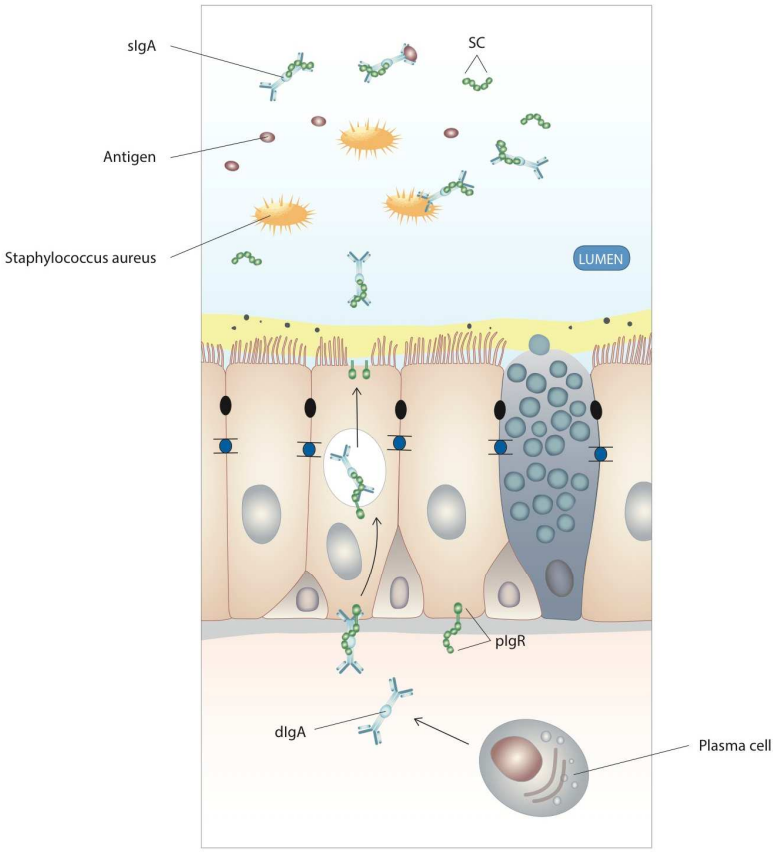

Figure 1 : pIgR and sIgA in the respiratory epithelium

Dimeric IgA (dIgA) is secreted by the plasma cells of the lamina propria. It binds to the pIgR at the basolateral pole of secretory epithelial cells. dIgA is transported through the epithelial cell to the apical surface, where a proteinase cleaves the large extracellular domain of the receptor, thereby releasing it, bound to dIgA, into the lumen. dIgA, in association with the cleaved receptor fragment (also known as secretory component, SC) form sIgA. In secretions, sIgA interacts with antigens/pathogens, neutralizing their ability to cause disease.

Local production of IgA in the sinonasal mucosa is well established since the early sixties[7,8]. However, very few has been published regarding IgA in different sinonasal pathologies. Bass et al. studied the localization and production of IgA in nasal mucosa and nasal polyps using immunofluorescent techniques[9]. They showed that bright staining IgA plasma cells localized around glandular elements were secreting their immunoglobulin contents into the gland lumens, with no difference in the localization or production of IgA between control nasal mucosa or nasal polyps. Van Zele et al. showed high levels of IgA in tissue homogenates from patients with CRSwNP, not correlated with serum IgA, suggesting a local production[10]. Similarly, Tan et al. showed an increase of total IgA levels in nasal polyp tissue extract relative to levels seen in CTRL nasal tissue extracts[11]. However, it remains unclear whether this increased IgA production translates into increased levels into sinonasal secretions.

Another study has shown an increase of specific IgA level in lavage fluid in patients with allergic rhinitis to Dermatophagoides pteronyssinus after nasal allergen provocation[12]. An increased expression of IgA receptors on eosinophils of allergic individuals has also been reported[13]. At the contrary, Hsin et al. examined the levels of S-IgA and total IgA in patients with allergic rhinitis, patients with chronic rhinosinusitis and control subjects, and showed no difference among the 3 groups[14].

The expression of pIgR/SC has been mainly studied in lower airway diseases. It is strongly decreased in severe chronic obstructive pulmonary disease (COPD)[2,15], correlated with airflow obstruction and associated to neutrophilic inflammation as neutrophil-derived proteinases are very potent to cleave both cell pIgR and soluble SC. The expression of pIgR/SC has

also been shown to be decreased in lung cancer[16]. In the upper airways, pIgR has been studied in patients with nasopharyngeal carcinoma, where it has been shown to be decreased[17] or mutated[18], with resultant decreased transcytosis of IgA, but little is known regarding its role in chronic sinonasal pathologies.

Because the upper and lower airways are linked physically, there has been an assumption that known defense mechanisms from the lower airways could be found also in the upper airways.

In contrast to the well-documented reduced epithelial expression of SC in COPD and lung cancer, this first line of defense of the respiratory tract mechanisms is virtually unexplored in chronic upper airway diseases.

The first part of this work concentrates thus on one of the first-line defense mechanisms of the airways, namely the S-IgA system, with a specific focus on its receptor, pIgR, and on the mucosal IgA response observed in chronic sinonasal diseases.

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Downregulation of polymeric immunoglobulin receptor and secretory IgA antibodies in eosinophilic upper airway diseases

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Abstract

Background: Immunoglobulin (Ig) A represents a first-line defence mechanism in the airways, but little is known regarding its implication in upper airway disorders. This study aimed to address the hypothesis that polymeric Ig receptor (pIgR)-mediated secretory IgA immunity could be impaired in chronic upper airway diseases.

Methods: Nasal and ethmoidal biopsies, as well as nasal secretions, were collected from patients with chronic rhinosinusitis (CRS) with (CRSwNP) or without nasal polyps (CRSsNP), allergic rhinitis (AR) and controls, and assayed for IgA1/IgA2 synthesis, pIgR expression, production of secretory component (SC), IgA and relevant IgA antibodies, and correlated to local eosinophils and inflammatory features (IL12, IL-13 and ECP).

Results: pIgR expression was decreased in the ethmoidal mucosa in CRSwNP (p=0.003) and in AR (p=0.006**).** This pIgR defect was associated with reduced levels of SC (p=0.007) and IgA antibodies to Staphylococcus aureus enterotoxin B (SAEB) (p=0.003) in nasal secretions from CRSwNP patients, and with increased IgA deposition in subepithelial areas. pIgR

downregulation was selectively observed in patients with tissue eosinophilia, whilst no clear relation to smoking history was observed.

Conclusion: Epithelial pIgR expression is decreased in patients with CRSwNP and AR, and results in decreased SC and IgA antibodies to certain bacterial antigens (SAEB) in nasal secretions of CRSwNP patients in parallel to subepithelial accumulation of IgA. This defect in mucosal immunity is associated with eosinophilic, Th2-related inflammation.

Abbreviations:

AR: Allergic rhinitis

COPD: chronic obstructive pulmonary disease

CRS: chronic rhinosinusitis

CRSsNP: CRS without nasal polyps

CRSwNP: CRS with nasal polyps

ECP: eosinophil cationic protein

Ig: immunoglobulin

pIgR: polymeric Ig receptor

SA : Staphylococcus aureus

SAEB : Staphylococcus aureus enterotoxin B

S-IgA: secretory Ig A

SC: secretory component

INTRODUCTION

Chronic rhinosinusitis (CRS) defines a group of disorders characterized by persistent inflammation of the sinonasal tract. The diagnosis is based upon the presence of persistent symptoms, nasal endoscopy and CT scan[1]. Depending on the presence of polyps on endoscopic examination, CRS **is** divided into 2 groups: CRS with (CRSwNP) or without nasal polyps (CRSsNP). Whereas the pathogenesis of CRS remains controversial, Th2-driven eosinophilic inflammation is classically observed in CRSwNP, in contrast to CRSsNP where neutrophils are predominant. Allergic rhinitis (AR) represents another Th2-related nasal disorder. The role of pathogens, for both inception and exacerbations, is supported by several studies[2, 3], whereas only a few report on defective defence mechanisms in these disorders[4-6].

Immunoglobulin (Ig) A is the predominant Ig in mucosal tissues, endowed with several protective functions such as neutralization of pathogens and particles[7]. At mucosal sites, plasma cells produce dimers of IgA, which are translocated across the epithelium from the lamina propria into mucosal secretions. This active transport is assumed by the polymeric immunoglobulin receptor (pIgR), expressed by epithelial cells, up to the apical pole where a proteolytic cleavage releases secretory IgA (S-IgA) consisting of d-IgA and the extracellular part of the pIgR known as secretory component (SC). This latter is thought to protect IgA from proteolysis and displays itself, independently from IgA, some anti-infectious properties.[8] IgA is secreted via this route into the mucous lining fluid of the gastrointestinal, urogenital and respiratory tracts, as well as into tears, saliva

and milk[9]. pIgR expression and function is regulated through multiple pathogen-associated molecular patterns and pro-inflammatory cytokines such as IFN-γ, IL-4, TNF-α, and IL-1 via transcriptional and post-transcriptional mechanisms[10, 11].

The expression of IgA and pIgR/SC has been mainly studied in the lung, where pIgR is decreased in severe chronic obstructive pulmonary disease (COPD) and in lung cancer.[7, 12] In the upper airways, pIgR is downregulated in patients with nasopharyngeal cancer, but little is known regarding IgA and pIgR expression in chronic inflammatory sinonasal pathologies.[13, 14] It has been shown that IgA is increased in tissue homogenates from patients with CRSwNP,[15, 16] but it remains to determine whether this results into increased IgA in nasal secretions. We hypothesized that pIgR could be downregulated in CRSsNP, often seen as the upper airway equivalent of COPD, and could result in reduced secretion of pathogen-specific IgA antibodies.

The aim of the present work was therefore to evaluate IgA synthesis and pIgR/SC expression in human nasal and sinusal tissues from patients with CRSwNP, CRSsNP and AR, as compared with controls, and to assess in nasal secretions from these patients SC and IgA to relevant bacterial antigens (S. pneumoniae and S. aureus), as well as their relation to eosinophilic and Th1(IL12p35) or Th2 (IL13)-related inflammation.

METHODS

For details, refer to the online supporting information section.

Subjects and surgical sampling

Patients with CRSsNP (n = 13), CRSwNP (n = 10), AR (n = 13) and controls (n = 20) were recruited at the outpatient clinic of our Department of Otorhinolaryngology (Cliniques Universitaires Saint-Luc, Belgium). The diagnosis of CRSwNP and CRSsNP was, accordingly to EPOS criteria, based on history, clinical examination, nasal endoscopy and CT scanning[1]. AR was diagnosed based on symptoms and positive skin prick tests and/or serum specific IgE (> 0.35 kU/L) to common aeroallergens, following the ARIA guidelines[17]. Clinical characteristics of the patients are described in Table 1. Controls and AR patients underwent surgery for anatomical obstruction, whilst CRS patients underwent surgery as indicated by the disease. All patients were weaned of oral and nasal corticosteroids or antibiotics for at least 3 weeks before surgery.

	Controls	**CRSsNP**	**CRSwNP**	**AR**
N	20	13	10	13
Sex (M/F)	13/7	8/5	8/2	8/5
Age, years (range)	38.6 (18-62)	35.7 (18-51)	48.2 (28-70)	35.4 (20-48)
History of asthma	1	0	3	2
Atopy	0	0	5	13
Smoking	4 (20%)	4 (30%)	3 (30%)	3 (23%)
CT score (Lund-Mackay)	1 (0-2)	12 (7-21)***	18 (14-24)***	1 (0-2)
Serum IgA (mg/dl)	228 (89-633)	233 (113-485)	207 (97-367)	185 (173-289)

Table 1. Patients characteristics.

Patients were assigned to disease groups based on criteria described in Methods, and associated features (asthma, atopic background, cigarette smoking) are stated. *** $p < 0.0001$ (Mann-Whitney U test) for CT scores, as compared to control subjects.

Sinonasal biopsies were taken from the inferior turbinate and anterior ethmoidal sinus**.** Nasal secretions were collected as previously described[18] for assessments of soluble factors including IgA and SC. Biopsies were processed for immunohistochemistry or PCR. Serum was also sampled to measure serum IgA.

All subjects gave signed informed consent, and the study was approved by the local ethical committee (Cliniques Universitaires Saint-Luc, Brussels).

Staining for pIgR, IgA and eosinophils

Paraffin-embedded sections of turbinal and ethmoidal tissues were incubated with rabbit anti-human SC IgG prepared as previously reported[19] and mouse anti-human IgA (ThermoScientific). To quantify pIgR and IgA staining, immunostained sections were scanned and analyzed using the Framework for Image Dataset Analysis (FrIDA).[20] The percentage of stained area was calculated in 10 epithelial (pIgR) or subepithelial (IgA) fields, at x400 magnification, and reported to the surface of examined area. Eosinophils were counted after hematoxylin & eosin staining in 10 epithelial fields, at x400 magnification. The presented data consists of the mean number of eosinophils per high power (x400) field.

Quantitative RT-PCR for pIgR, IgA1, IgA2 and cytokines

Two biopsies per patient (1 ethmoidal, 1 turbinal) were processed for gene expression by RT-qPCR. The expression levels of pIgR, IL12p35 and IL13 in sinonasal tissue were quantified by RT-qPCR using the iCycler IQ5 PCR thermocycler (Bio-Rad, USA) and reported to those of RPS18 housekeeping gene. Absolute quantification of IgA1 and IgA2 expression levels was

determined with RT-qPCR using an ABI PRISM 7900 Sequence Detection System thermal cycler according to manufacturer's instructions (Applied Biosystems, USA).

Immunoassays for IgA, SC, and ECP

Concentrations of IgA, SC, IgA1, IgA2, specific IgA antibodies to pneumococcal anticapsular and S. aureus enterotoxin B (SAEB) antigens were determined in nasal secretions by ELISA. Eosinophil cationic protein (ECP) was measured by ImmunoCAP (Thermofisher) following manufacturer's instructions.

Statistical analysis

Data are expressed in dot plots and presented as median and interquartile range. Statistical analysis was performed by the Kruskal–Wallis and Mann–Whitney U two-tailed tests for unpaired comparisons. When multiple between-group comparisons were made, the Kruskal–Wallis test was used to establish the significant intergroup variability, and the Mann–Whitney U-test was then used for between-group comparisons. Data were correlated by the Spearman rank test. A P level < 0.05 was considered as significant.

RESULTS

pIgR expression in the sinonasal mucosa

pIgR mRNA expression was decreased in the ethmoidal mucosa from CRSwNP and AR patients, when compared to controls (Figure 1A), whilst it was not reduced in CRSsNP. No significant change in sinonasal pIgR expression was observed in relation to cigarette smoking (not shown). This downregulation in pIgR transcription was only observed in the ethmoidal, and not in the turbinal mucosa from these patients.

At the protein level, pIgR immunodetection was confirmed in ciliated and seroglandular cells from the surface epithelium and submucosal glands (Figure 1C). No staining was found in basal cells and in goblet cells. Quantification of pIgR protein confirmed a significant reduction in CRSwNP and AR (Figure 1B).

Total IgA and SC in nasal secretions and sinonasal mucosa

We assessed whether pIgR downregulation resulted in decreased IgA levels in nasal secretions and sinonasal mucosa.

First, SC concentration in nasal secretions was accordingly reduced in CRSwNP (Figure 1D) and correlated with tissue pIgR mRNA levels (Figure 1E).

In order to assess each compartments of the airway mucosa, we evaluated IgA content in sinonasal tissue by IgA immunostaining. A significant

accumulation of IgA was observed in subepithelial areas of ethmoidal tissue from CRswNP (Figure 2A).

IgA synthesis was then assessed by RT-qPCR for IgA1 and IgA2 transcripts in sinonasal tissues, which showed no significant changes among the different groups (Figure 2B). Similarly, levels of IgA and IgA subclasses in nasal secretions were not significantly affected (Figure 2C). Of note, serum IgA was not significantly different among the groups (Table 1).

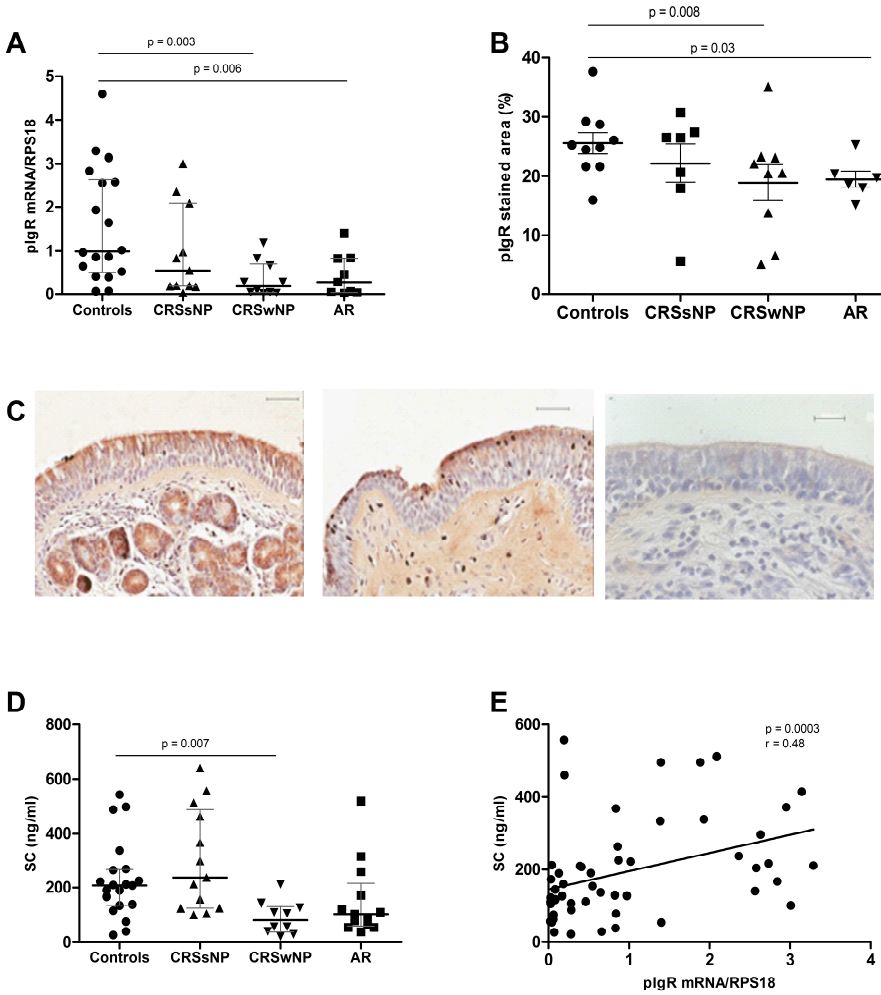
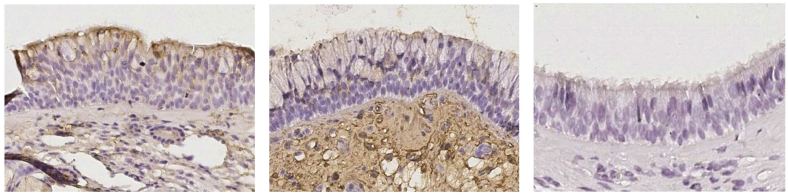
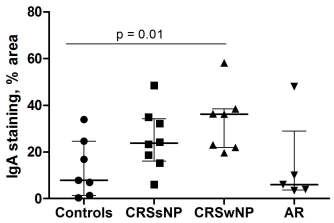


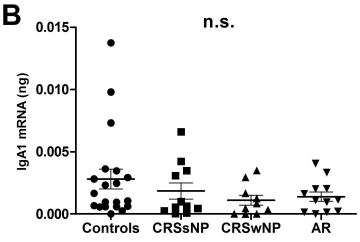
Figure 1: pIgR/SC expression in patients with upper airway disease. pIgR mRNA expression (A) in the ethmoidal mucosa from patients with CRSwNP, CRSsNP and AR, as compared to controls. pIgR protein detected by IHC was quantified in sinonasal tissue (B) and is shown (C) in tissue from one representative control and one representative CRSwNP patient, as compared to negative control with rabbit IgG (left, medium and right panel, respectively). Secretory component (D) production in nasal secretions. Correlation of pIgR mRNA expression in the ethmoidal mucosa to SC in nasal secretions according to Spearman's method (E). Bar represents 50µm. *P* values according to Kruskal-Wallis followed by Mann-Whitney tests.

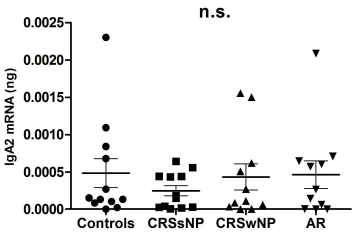
A



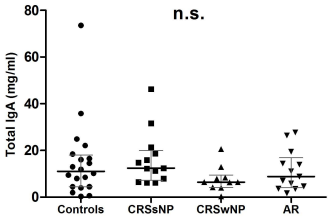

p = 0.01
IgA staining, % area
0
20
40
60
80
Controls
CRSsNP
CRSwNP
AR

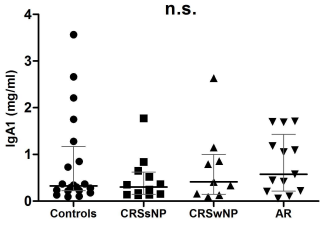
B


n.s.
IgA1 mRNA (ng)
0.000
0.005
0.010
0.015
Controls
CRSsNP
CRSwNP
AR


n.s.
IgA2 mRNA (ng)
0.0000
0.0005
0.0010
0.0015
0.0020
0.0025
Controls
CRSsNP
CRSwNP
AR

C


n.s.
Total IgA (mg/ml)
0
20
40
60
80
Controls
CRSsNP
CRSwNP
AR


n.s.
IgA1 (mg/ml)
0
1
2
3
4
Controls
CRSsNP
CRSwNP
AR

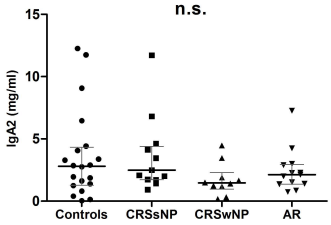

n.s.
IgA2 (mg/ml)
0
5
10
15
Controls
CRSsNP
CRSwNP
AR

Figure 2: **IgA expression in patients with upper airway disease**. IgA detected by IHC is shown in tissue from one representative control and one representative CRSwNP patient, as compared to negative control with mouse IgG (upper left, right and lower left panel, respectively), and was quantified in subepithelial areas (A). IgA1 and IgA2 mRNA expression (B) in the ethmoidal mucosa from patients with CRSwNP, CRSsNP and AR, as compared to controls. IgA1, IgA2 and total IgA (C) in nasal secretions from the same patient groups as shown in Figure 1. *P* values according to Kruskal-Wallis followed by Mann-Whitney tests.

Specific IgA antibodies in nasal secretions

We next assessed specific IgA antibodies to S. pneumonia and S. aureus antigens. Whereas non-significant changes were observed for S. pneumoniae, SAEB-specific IgA was significantly decreased in nasal secretions from patients with CRSwNP (Figure 3).

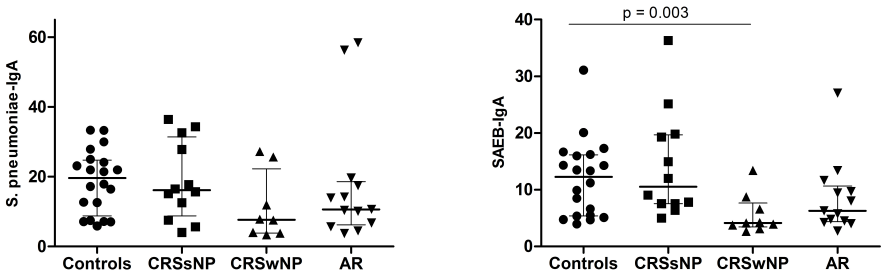


Figure 3: **Specific IgA antibodies to S. aureus and S. pneumonia antigens in patients with upper airway disease.** Specific IgA antibodies to pneumococcal anticapsular antigens and to Staphylococcus aureus enterotoxin B (SAEB) in nasal secretions from the same patient groups as shown in Figure 1. Results are expressed in arbitrary units. *P* values according to Kruskal-Wallis followed by Mann-Whitney tests.

pIgR expression and eosinophilic inflammation of the sinonasal mucosa

As pIgR/SC and SAEB-IgA were decreased in CRSwNP, which is reportedly associated with eosinophilic inflammation, we addressed the relation between pIgR downregulation and eosinophilic inflammation in our patients.

Increased numbers of eosinophils were observed, as expected, in subepithelial ethmoidal tissues from patients with CRSwNP (Figure 4A). Eosinophils were also observed in a subset of patients with CRSsNP.

When dichotomizing patients according to the presence or not of high eosinophil numbers (i.e. ≥2 versus <2 eos per h.p. field), pIgR downregulation was selectively observed in patients with eosinophilic upper airway disease (Figure 4B). In order to further assess a correlation between eosinophils and pIgR expression, ECP was measured in nasal fluid. ECP was significantly increased in patients with AR, but not CRSwNP (Figure 4C), and no correlation was observed with pIgR expression (p= 0,5; r= -0.1).

Cytokine expression in the sinonasal mucosa

IL-13 mRNA was significantly increased in the ethmoidal mucosa of CRSwNP, as well as to a lower extent in CRSsNP (Figure 4D). IL-13mRNA was correlated to eosinophil numbers in ethmoidal biopsies (p=0.001, r=0.48), indicating a Th2-related immune response.

In contrast, IL12p35 expression was only increased in the ethmoidal mucosa of CRSsNP patients (Figure 4E). In addition, IL-12p35 mRNA was correlated with pIgR expression (Figure 4E, right panel).

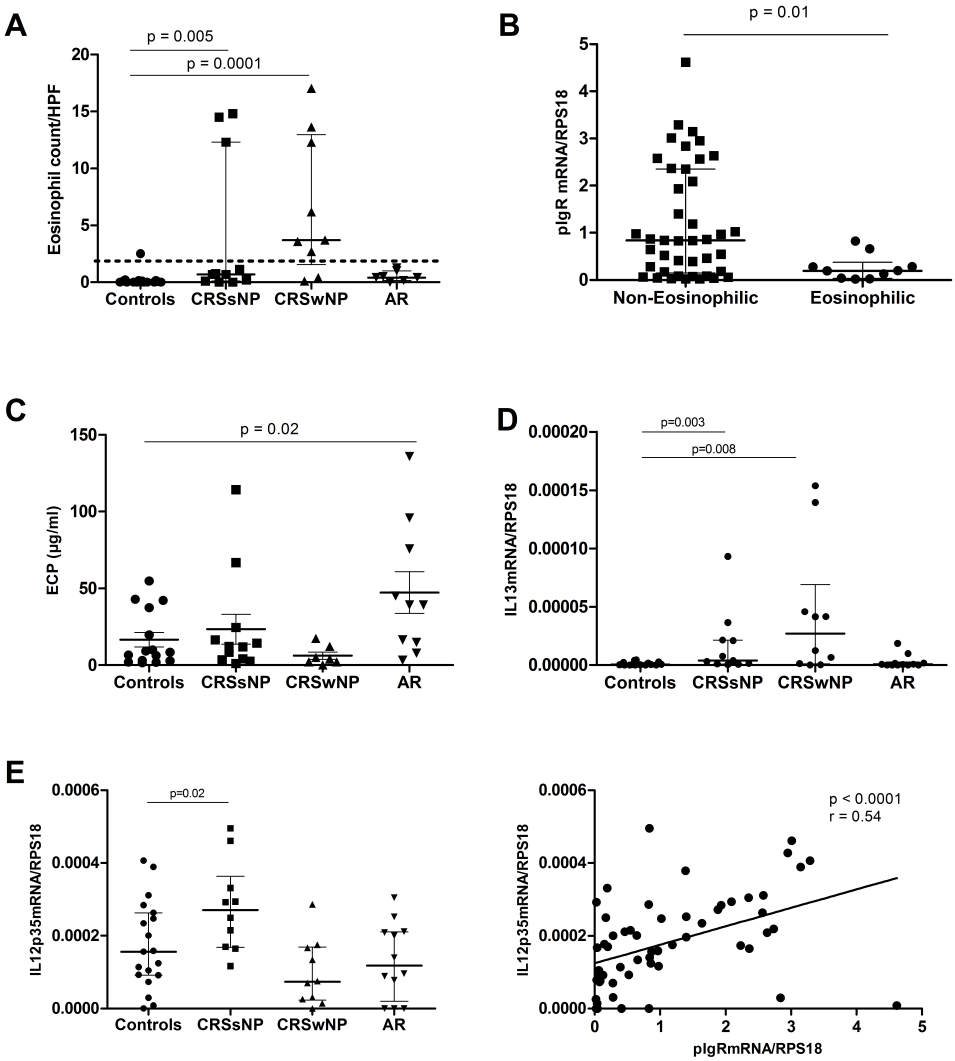


Figure 4: Eosinophils, ECP and and expression of IL-13 and IL-12 in patients with upper airway disease. Eosinophils (expressed as n cells per h.p. field) in the ethmoidal mucosa from patients with CRSwNP, CRSsNP and AR, as compared to controls (A), and pIgR expression is shown in these patients plotted as 'eosinophilic' (≥2 eos per h.p. field) versus low/non eosinophilic (<2**) (B**). Production of ECP in nasal fluid (C), and expression of IL-13 mRNA (D) and IL-12p35 mRNA (E) in the ethmoidal mucosa from with

CRSwNP, CRSsNP and AR, as compared to controls, and correlation with pIgR mRNA (right panel) according to Spearman's method. *P* values according to Kruskal-Wallis followed by Mann-Whitney tests.

DISCUSSION

This study is, to our knowledge, the first to investigate epithelial pIgR in human chronic inflammatory disorders of the upper airways. It shows that the local expression of pIgR is downregulated in patients with CRSwNP and AR, but not in CRSsNP. This epithelial defect in CRSwNP resulted in the accumulation of IgA in subepithelial tissue (while active synthesis of IgA1 and IgA2 was unaffected) and in reduced SC and SAEB-specific IgA antibodies in nasal secretions. Moreover, correlation to immuno-inflammatory features highlighted that whilst pIgR positively correlates to IL-12 expression as Th1 signature, pIgR downregulation was closely related to Th2-type eosinophilic inflammation of the ethmoidal mucosa.

In the upper airways, pIgR has been studied in nasopharyngeal carcinoma, where it has been shown to be decreased or mutated, with resultant decreased transcytosis of IgA[13, 14]. Our data show that pIgR is well detected in the human sinonasal epithelium. Contrary to our original hypothesis, pIgR was selectively downregulated in the ethmoidal tissue of CRSwNP and AR, and was not affected in the turbinal mucosa. We also showed that pIgR defect results into reduced secretion of SC in CRSwNP. In contrast, the tissue content of IgA was increased in subepithelial areas from patients with CRswNP, while active synthesis was not affected. These findings indicate that increased IgA content reported in previous studies of tissue homogenates from patients with CRSwNP[15] does not relate to increased IgA

production but probably to the aborted IgA transport into secretions due to impaired epithelial pIgR expression.

The pIgR defect in CRSwNP resulted in very modest and not significant reduction in IgA(2) in nasal secretions. However, levels of IgA antibodies to SAEB were significantly reduced in nasal secretions from these patients. Thus, as the pIgR assures the transport of IgA irrespectively of its specificity, its reduced expression in the ethmoidal epithelium from CRSwNP is not sufficient to drive significant decreases in "total" IgA, although a trend was observed (Figure 2C), in contrast to secretory component which was clearly decreased. IgA in secretions may arise locally, from synthesis by local plasma cells and subsequent pIgR-mediated transport, but also from exudation from the systemic circulation. As CRSwNP is associated with intense local inflammation and edema, we hypothesize that the latter element may "restore" the IgA levels in secretions from these patients, but does not compensate for reduced transport of locally produced IgA antibodies to S. aureus enterotoxin. An alternative possibility for the apparent discrepancy between reduced levels of SAEB-specific IgA and preserved levels of total IgA could lie in a specific defect in the generation of IgA antibodies to SAEB, which appears less likely.

The finding of a defect in specific IgA immunity to this S. aureus superantigen is interesting considering the probably important role of SAEB in driving IgE-mediated inflammation in CRSwNP, where a proTh2 role of SAEB has been identified.[21] Given the major role of IgA in frontline defence of the airways, including inhibition of the adherence of pathogens to the epithelium (so-called "immune exclusion") and neutralization of pro-inflammatory cytokines such as CXCL8/IL-8, it may be speculated that

reduced pIgR-mediated immunity could affect first line and specific immunity in (eosinophilic) CRS and AR, favoring bacterial colonization and leading to a vicious circle of impaired anti-bacterial immunity and secondary chronic inflammation[8, 22]. The higher colonization by S. aureus in CRSwNP could be partly explained by decreased specific IgA to SAEB, as IgA plays an important role as a local scavenger of inhaled antigens. Moreover, IgA antibodies may probably also act as scavengers for allergens, which are reportedly defective in patients with AR and upregulated following allergen-specific immunotherapy[23]. This post-immunotherapy IgA response was shown to be selective for the IgA2 subclass and induced monocyte IL-10 secretion, whilst inhibiting IL-12 expression[23,24]. In addition to Fab-mediated antigen neutralization, a defect in S-IgA antibodies could also result in reduced (Fc-mediated) opsonisation of pathogens. As a defect in the phagocytosis capacity of macrophages from CRSwNP patients has been observed[6], it remains to explore whether reduced cytophilic S-IgA antibodies could contribute to this observation. Moreover, interplay between S. aureus and ECP has been recently observed, i.e. for the regulation of receptor for advanced glycation end products[25]. Our data further support a relationship between eosinophilic inflammation and local immunity to pathogen-derived products. Although balanced by the known degranulating effect of secretory IgA on eosinophils, these mechanisms could further indicate that IgA contributes via several pathways to control mucosal inflammation[26].

In the lung, pIgR deficiency has been observed in the bronchial epithelium from smokers who developed severe COPD, and linked to neutrophil infiltration of submucosal glands[7, 12]. In contrast, our data show that pIgR

downregulation is not clearly related to smoking but rather to eosinophilic inflammation. Eosinophilia was as expected a common feature in CRSwNP, but was also observed in some CRSsNP patients[27-29]. We observed that tissue eosinophils were more discriminant than the upper airway disease group, in identifying patients with pIgR downregulation, suggesting that this pIgR defect closely relates to eosinophilic inflammation. In contrast, a significant correlation was found between pIgR and IL12p35 expression. As IL-12 is a key cytokine for the induction of IFN-γ-producing Th1 cells, this observation could be consistent with the known stimulatory role of IFN-γ on pIgR synthesis.[30] However, it has been shown that IL-4 may also upregulate pIgR transcription, in synergy with IFN-γ.[31] As eosinophilic upper airway disorders are associated with Th2-type immune responses, it could be speculated that besides IL-4 other Th2-related factors could drive pIgR downregulation. Thus, our positive correlation between pIgR expression and IL-12 may suggest that, in addition or alternatively to favoring eosinophilic inflammation by limiting the scavenging effect of IgA antibodies to bacterial (super) antigens, pIgR downregulation could also result from the effect of mediators of Th2-type eosinophilic inflammation. Although this needs to be confirmed, notably using nasal epithelial cells, the putative pathways linking pIgR downnregulation and eosinophilic upper airway disease are summarized in Figure 5.

Altogether this study shows for the first time that pIgR is well expressed in the human sinonasal mucosa, especially in seroglandular and ciliated epithelial cells, and that its expression is decreased in patients with CRSwNP and AR. This defect results in reduced secretion of SC and specific IgA antibodies to SAEB in nasal fluid from patients with CRSwNP**,**

as well as in subepithelial accumulation of IgA**.** These data also indicate a previously unrecognized link between eosinophilic inflammation and pIgR-mediated secretory immunity, suggesting that pIgR impairment is observed in the eosinophilic phenotype of upper airway disease.

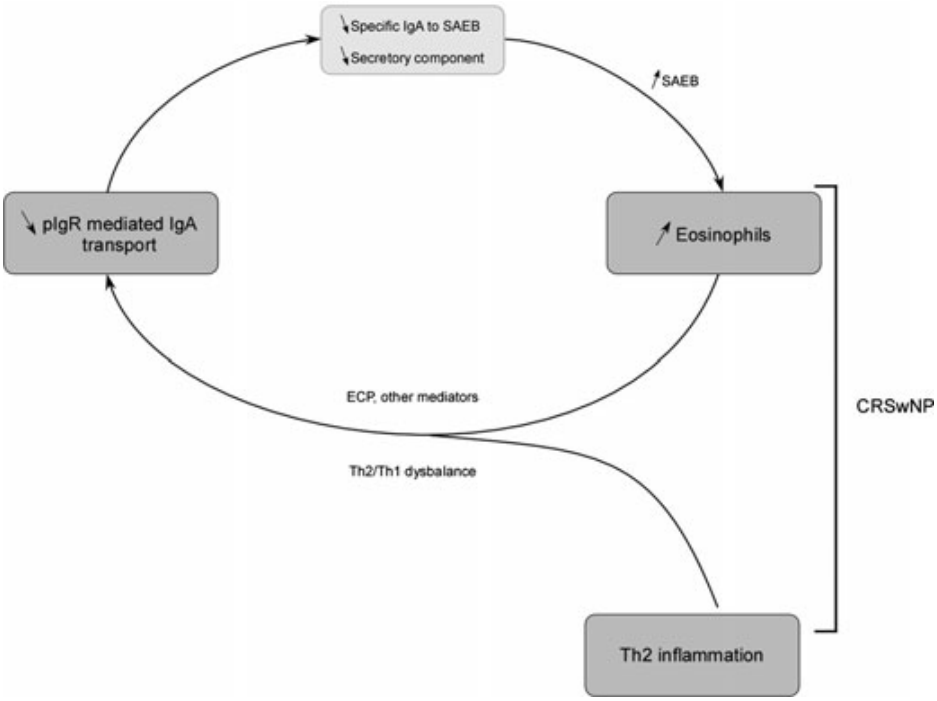


Figure 5: **Putative pathways linking decreased pIgR expression and eosinophilic inflammation in CRSwNP.** The proposed mechanisms underlying the relationship between pIgR-dependent IgA immunity and eosinophilic inflammation of the upper airways, include decreased pIgR expression resulting in reduced IgA-mediated neutralization of some pathogens and/or antigens (e.g., S. aureus enterotoxin B), and in turn favoring eosinophilic inflammation (e.g. favored by SAEB-IgE). Conversely, it may also result from the effect of local mediators of eosinophil/Th2-related airway inflammation on the sinonasal epithelium.

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Supporting information

Downregulation of polymeric immunoglobulin receptor and secretory IgA antibodies in eosinophilic upper airways diseases

Methods

Immunohistochemistry for pIgR and IgA

After surgical removal, the turbinal and ethmoidal biopsies were immediately immersed in 4% formaldehyde in phosphate-buffered saline at pH 7.4 for at least 24 h. Samples for immunohistology were embedded under vacuum in paraffin. Serial sections of 5 μm thickness were cut from paraffin blocks, spread on polylysine-coated glass slides, and dried at 40° C for at least 24 h.

The slides were then processed for immunostaining, each step of the procedure being followed by washing with Tris-buffered saline (pH 7.4). After disembedding and rehydration of the specimen, endogenous peroxidases were inhibited by incubation in 0.03% (vol/vol) H_2O_2 in water for 30 min, and the slides were treated with 1% (wt/vol) BSA in Tris-buffered saline for 30 min to neutralize remnant reactive aldehyde groups originating from fixation. Slides were then incubated overnight at 4° C with rabbit anti-human SC IgG prepared as previously reported[1] and mouse anti-human IgA (ThermoScientific). Control sections were treated with rabbit anti-chicken IgY/mouse IgG at the same dilution. The secondary antibody, (biotinylated goat anti-rabbit IgG (Sigma, 1/1000), biotinylated rabbit anti-mouse IgG (Sigma, 1/3000) was applied in 10% (wt/vol) defatted dry milk

for 30 min. The reaction was amplified with streptavidin–peroxidase conjugate (BD Pharmingen, 1/500) in Tris-buffered saline containing 1% BSA for 30 min, and color was developed by incubation with diaminobenzidine in 0.03% H_2O_2 for 10 min. After the reaction was stopped by washing in water, slides were counterstained with Mayer's hemalum and mounted with coverslips in Eukitt's medium. Images of each section were captured by using an Aperio ScanscopeAT scanner.

To assess pIgR and IgA quantification, unmodified images of scanned turbinal and ethmoidal sections were analyzed using the Framework for Image Dataset Analysis (FrIDA), a custom open source image analysis tool from the Johns Hopkins University, as previously described.[16] Briefly, color masks were generated to identify DAB brown. A meta mask created to include all brown pixels in the epithelial (pIgR)/subepithelial (IgA) zone was used to analyze each image. We calculated the percentage of positive area fraction in ten epithelial/ subepithelial fields per section, at x400 magnification.

RT-PCR for pIgR, IL12p35, IL-13 and IgA1/IgA2 mRNA expression

Total RNA was isolated using the Rneasy® Plus Mini kit (Qiagen). Each 500 ng of total RNA was reverse- transcribed with RevertAidTM Reverse transcriptase kit (Fermentas) with 0.3µg of random hexamer (Invitrogen) and 1mM of each dNTP (Invitrogen) following to the manufacturer's protocol in a thermocycler (Applied Biosystem). The expression levels were quantifed by real time quantitative PCR with the iCycler IQ5 PCR (Bio-Rad). The reaction mix contained 5 µl of cDNA diluted 10-fold, 400nM of

each primer (pIgR reverse: CAG CCG TGA CAT TCC CTG ; pIgR forward: CTC TCT GGA GGA CCA CCG T ; RPS18 reverse: TGA TCA CAC GTT CCA CCT CAT ; RPS18 forward: TGT GGG CCG AAG ATA TGC T; IL-13 forward: CTG GAA TCC CTG ATC AAC GT . IL-13 reverse: GAA AAC TGC CCA GCT GAG AC) and 2x iQTM SYBR®Green Supermix (Bio-Rad) in a final volume of 20µl. The cycling conditions were 95°C for 3 min followed by 40 cycles of 95°C for 15s and 60°C for 30s. To control the specificity of the amplification products, a melting curve analysis was performed. Moreover, the negative controls (water and –RT) showed no amplification. Samples were run in duplicate and the copy number was calculated from a standard curve. The standard curve and data analysis were produced using Bio-Rad iQ5 Software.

For IgA1 and IgA2, PCR was performed by using an ABI PRISM 7900 Sequence Detection System thermal cycler according to manufacturer's instructions (Applied Biosystems, Life Technologies Corporation, California, USA) using the equivalent of 1.25ng reverse transcribed RNA per reaction. Gene specific primer/probe sets were designed using the on-line Universal ProbeLibrary Assay Design Centre (Roche Applied Science, Burgess Hill, UK). Universal ProbeLibrary probes were purchased from Roche Applied Science and corresponding forward and reverse primers synthesized by Sigma (Sigma-Aldrich Company Ltd, Dorset, UK). The primer/probes sets were as follows: IgA1, Forward-CAT GCCACGTGAAGCACTAC, Reverse-GGTAGGTGGAGTTGAGGGAAC T, Probe-UPL Probe 20; IgA2, Forward-CGCCAACATCACAAAATCC, Reverse-CGTCACCAGCTCGTTCAG, Probe-UPL Probe-17.

SDS software was used to determine the absolute quantification of the target cDNA. Standard curves were created using plasmids containing cDNA for IgA1, I.M.A.G.E cDNA clone 4701069 and IgA2, I.M.A.G.E cDNA clone 4765168 (Source Bioscience UK Limited, Nottingham, UK).

ELISA for SC and IgA

SC, IgA, IgA1 and IgA2 concentration were determined in nasal secretions by a specific enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well microplates were coated with 1 μg/ml affinity-purified goat anti-SC antibody (developed in our laboratory, recognizing both soluble SC and membrane pIgR/SC) in 0.1 M carbonate-bicarbonate buffer, pH 9.6, overnight at 4°C. After washings with 1/1.000 vol/vol Tween 20-PBS (PBST) and blockade with 1% wt/vol BSA in PBST for 1h at 37°C, samples and serial dilutions of purified human soluble SC/IgA1/IgA2 were incubated for 2 h at 37°C. After washings with PBST, plates were then incubated with biotinylated goat anti-SC/ biotinylated mouse anti-IgA1/ biotinylated mouse anti-IgA2 for 2 h and washed in TBST. A volume of 100 μl streptavidin–polyHRP in a 1:10.000 dilution in HPE buffer was added to each well and incubated for 30 min at room temperature. The plates were washed three times using PBST, and 100 μl of (3,5,3′,5′)-Tetramethylbenzidine substrate was added to each well. After incubation, the reaction was stopped by the addition of 100 μl of 2 M H_2SO_4. The absorbance was measured at 450 nm.

Total IgA concentration was determined by the same method (coating with 1 μg/ml affinity-purified goat anti-SC antibody, blockade with 1% wt/vol BSA in PBST, samples and serial dilutions purified total IgA) except for the

secondary antibody which was anti-human IgA (α-chain specific)−peroxidase antibody produced in goat. As it was peroxidase-conjugated, use of streptavidin–polyHRP was not necessary.

To assess specific IgA to pneumococcal anticapsular antigens and Staphylococcus aureus enterotoxin B, the same method was used, adapted from a previous report.[2] Microplates were coated with the whole 23-valent pneumococcal polysaccharide vaccine (Sanofi-Pasteur MSD) or with Staphylococcal Enterotoxin B from Staphylococcus aureus (Sigma-Aldrich). The detection antibody was HRP-conjugated goat anti-human IgA (α-chain specific). Results were expressed as arbitrary units, by reference to serial dilutions of a highly positive sample (OD given by 1:2 dilution set as 1 unit, and corrected to the dilution factor).

Immunoassay for ECP

Quantification of ECP was carried out on nasal fluid samples by ImmunoCAP assay (Thermofisher), according to the manufacturer's instructions.

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CHAPTER 4 : DOES THE SINONASAL EPITHELIUM UNDERGOES DE-DIFFERENTIATION IN CHRONIC UPPER AIRWAY DISEASES AND, IF SO, IS IT ASSOCIATED WITH EMT AND CHANGES IN LINEAGE SPECIFICATION?

INTRODUCTION

Does the sinonasal epithelium undergo de-differentiation in chronic upper airway diseases and, if so, is it associated with EMT and changes in lineage specification?

In the previous chapter, we thus showed that pIgR was downregulated in CRS. In parallel, our group showed that this pIgR expression is intimately related to epithelial differentiation[1]. Indeed, in COPD, it has been shown that reduced pIgR expression is secondary to altered epithelial cell differentiation and could lead to defective mucosal immunity, potentially contributing to a pathologic cycle of chronic airway inflammation and progressive remodeling[2].

As it is also known that remodeling is another major feature of CRS[3, 4], we thus wondered whether pIgR downregulation could be due to a dedifferentiation of the upper airway epithelium, involving the activation of the epithelial-mesenchymal trophic unit, which controls the local tissue microenvironment.

It is known that nasal epithelial repair is a well-coordinated and highly organized process, involving cell proliferation, differentiation and matrix degradation and deposition, regulated by a wide variety of growth factors and cytokines[5]. In-vivo and in-vitro studies showed that, during normal epithelial turnover and repair, undifferentiated basal cells (BC) progressively differentiate into ciliated and goblet cells (CC and GC), to reconstitute a functional respiratory epithelium[6, 7]. Wound healing may either restore the epithelium ad integrum or induce remodeling of the

airways, resulting in chronic inflammation and altered differentiation[8]. The first step of the repair process requires loss of differentiation (or de-differentiation), spreading, and migration of airway epithelial cells from the edges of the wound[9], while the second step is epithelial redifferentiation into CC or GC.

One of the best examples of cell de-differentiation is epithelial-to-mesenchymal transition (EMT), which has been observed in epithelial tissues in response to stress or injury, contributing to fibrosis in the kidney,[10] liver,[11] and lung[12] both in human and animal models.

What is EMT?

From a general perspective, epithelial-mesenchymal transition (EMT) is about disaggregating epithelial units and reshaping epithelia for movement[13]. More specifically, EMT is a biologic process that allows polarized epithelial cells, which normally interact with basement membrane (BM) via their basal surface, to undergo multiple biochemical changes that enable them to assume a mesenchymal cell phenotype, which includes the lost of cell-cell polarity and adhesion, resulting in enhanced migratory capacity, the downregulation of junction proteins and the modulation of their cytoskeleton organization, with acquisition of vimentin filaments[13].

EMT is an essentiel mechanism during embryonic development, as without mesenchymal cells, tissues and organs would never be formed. EMT is required for gastrulation and morphogenesis of the neural crest, musculoskeletal system, craniofacial structures, and peripheral nervous system[14]. The epithelium can also undergo EMT in adult physiological

processes, such as wound healing[15] or inflammation[16], leading to fibroblast production and fibrogenesis. Finally, EMT occurs in neoplastic cells, in the context of tumor growth and cancer progression[17].

One of the main distinguishing characteristics of EMT is the loss or down-regulation of adherens junctions and cytokeratin intermediate filaments.

The adherens junction complex of epithelial cells, which show disturbed localization in some inflammatory conditions, serve as an important paracellular barrier in polarized epithelial cells.[18] As the airway epithelium is the natural barrier between the environment and underlying tissue, its integrity is important for protecting the airways against noxious inhalants, such as environmental tobacco smoke, particles and biological agents. Damage to respiratory epithelium is known to contribute to the pathogenesis of airway diseases through the alteration of barrier function and permeability of the mucosa.[19] Therefore, regulation of cell–cell junction stability and dynamics is crucial to maintain tissue integrity and allowing tissue remodelling throughout development. The cadherin family, among others, mediates cell-cell adhesion. E-cadherin is one of the best-characterized adherens junctions expressed on nasal epithelium.

A defective epithelial barrier was found in patients with AR after Der p 1 antigen exposure along with a decreased expression of tight junctions proteins, contributing to increased epithelial permeability and representing thereby a potential mechanism for transepithelial antigen exposure in AR[20].

A disruption of epithelial integrity with reduced expression of tight junctions protein has also been shown in CRSwNP [21-23].

Vimentin is the most ubiquitous intermediate filament protein and the first to be expressed during cell differentiation. All primitive cell types express

vimentin but in most non-mesenchymal cells, other intermediate filament proteins replace it during differentiation.

The up-regulation of vimentin expression is a particularly wellknown marker of the EMT, and even appears to be a prerequisite for EMT induction.Vimentin contributes to EMT via upregulating the gene expression of several EMT-linked genes[24]. Only a few studies have been published regarding vimentin in chronic upper airway diseases. Its presence is confirmed in sinonasal mucosa from control and CRS patients[25]. Meng et al. showed a trend to a higher, although non-significant number of vimentin+ cells in the turbinate part of nasal polyps, as compared to normal nasal mucosa[22].

Epithelial cells undergoing EMT thus change their intermediate filament status from a keratin-rich network that connects to adherens junctions, to a vimentin-rich network connecting to focal adhesions. Concretely, they lose epithelial makers, such as E-cadherin and cytokeratins, but express mesenchymal markers, such as vimentin.

This process has been widely described in pathologic conditions of the lung, such as lung fibrosis[12] or obliterans bronchiolitis[26] whilst it remains debated in COPD[27] and asthma[28].

Emerging evidence suggests that the upper airway epithelium also could contribute to airway remodeling through the process of EMT. The aim of this second chapter was thus to describe more specifically the changes that the upper airways epithelium undergoes in terms of terminal and lineage differentiation during chronic sinonasal disesaes, in order to better understand the mechanisms leading to inflammation and remodeling.

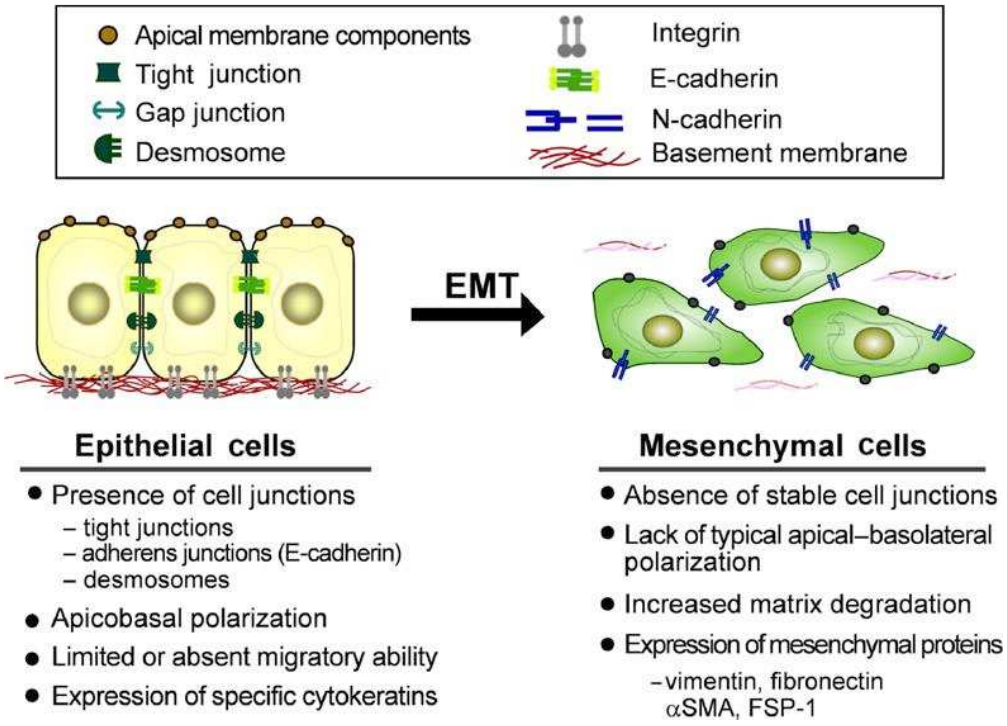

Figure 1 Major features of epithelial and mesenchymal cells. Epithelial cells contain specialized junctional proteins, exhibit apico-basal polarity, and have limited potential for dissociation and migration. In contrast, mesenchymal cells do not form specialized adhesion complexes and are irregular in shape with end-to-end polarity and focal adhesions resulting in increased migration capacity. During EMT, epithelial cells gain mesenchymal features which include changes in the expression of epithelial and mesenchymal markers (from Lee and Nelson, New Insights into the Regulation of Epithelial–Mesenchymal Transition and Tissue Fibrosis, Int Rev Cell Mol Biol. 2012;294:171-221.)

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Features of mesenchymal transition in the airway epithelium from chronic rhinosinusitis

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Abstract

Background: Chronic rhinosinusitis (CRS) defines a group of disorders characterized by persistent inflammation of the sinonasal tract. Epithelial changes and structural remodelling are present, but whether epithelial differentiation is altered remains uncertain.

Methods: To evaluate the differentiation state of the sinonasal epithelium in CRS, sinonasal biopsies from patients with CRS with (CRSwNP) or without polyps (CRSsNP), or with allergic rhinitis (AR), as compared to controls, were processed by immunohistochemistry and RT-qPCR for terminal differentiation (E-cadherin, high molecular weight cytokeratins (Hmw CK) and CK5, vimentin) and lineage differentiation (ß-tubulin IV+ ciliated cells, MUC5AC+ goblet cells, p63+ basal cells). Findings were correlated to subepithelial fibrosis and clinical CT score.

Results: Expression of E-cadherin was decreased at protein and mRNA levels in CRSwNP and CRSsNP, as compared to controls. Staining for Hmw CKs was also reduced in CRSwNP and CRSsNP, and CK5 mRNA was decreased in CRSwNP. These features were not due to changes in lineage specification, but associated with increases in vimentin-expressing epithelial

cells. In addition, vimentin expression correlated with the basement membrane thickening and with CT score, as well as with tissue eosinophils.

Conclusion: Features of epithelial de-differentiation towards a mesenchymal phenotype are observed in CRSwNP and CRSsNP, and correlate with airway fibrosis and inflammation.

Abbreviations:

AR: allergic rhinitis

BCs: basal cells

BM: basement membrane

CCs: ciliated cells

CK: cytokeratin

COPD: chronic obstructive pulmonary disease

CRS: chronic rhinosinusitis

CRSsNP: chronic rhinosinusitis without nasal polyps

CRSwNP: chronic rhinosinusitis with nasal polyps

Hmw CK: high molecular weight cytokeratins

Ig: immunoglobulin

IL: interleukin

RT-qPCR: real time quantitative polymerase chain reaction

INTRODUCTION

Chronic rhinosinusitis (CRS) is defined as a chronic inflammation of the nose and the paranasal sinuses, characterized by persistent symptoms and typical features at nasal endoscopy and CT scan[1]. CRS has been typically divided into CRS with nasal polyps (CRSwNP) and without nasal polyps (CRSsNP) based on the presence or not of polyps on endoscopic examination, and differ from allergic rhinitis (AR), which represents another very common chronic nasal disease[2].

Besides inflammation, these chronic upper airways disorders are characterized by remodelling of structural components, especially of the epithelium. The upper airway epithelium provides both a physical and chemical barrier through the formation of a strong mechanical cohesion by apical (tight and adherens) junctions and by secretion of a large array of antimicrobial host defense molecules mediating innate immunity respectively. Tissue remodelling in upper airways is an active process that includes increased extracellular matrix deposition, with subepithelial basement membrane (BM) thickening[3].

The normal upper airway epithelium comprises three main cell types, namely ciliated cells (CCs), representing 50% to 90% of the airway epithelial cell population[4, 5], mucus-secreting goblet cells (GCs) and basal cells (BCs). BCs represent 6 to 30% of the epithelial population in the lower airways and are considered as stem cells[6]. GCs and CCs act in concert to support mucociliary clearance and remove inhaled potentially harmful substances. Changes in epithelial specification have been described in

chronic airway diseases and following injury. Thus the number of CCs is decreased in asthma[7], after a viral infection[8] or in response to environmental toxics such as cigarette smoke[9]. In contrast, changes in these epithelial lineages remain unclear in CRS, with controversial findings reported in CRSwNP and CRSsNP regarding MUC5AC expressing GCs[10, 11]. One study also reported increased BC numbers in CRSwNP[12].

Besides commitment of epithelial cells to ciliated or goblet phenotypes, terminal differentiation is critical to epithelial homeostasis. Upon injury, epithelial cells may de-differentiate through squamous metaplasia or so-called epithelial-to-mesenchymal transition (EMT), which describes a rapid and normally reversible modulation of the epithelial phenotype towards mesenchymal cells[13]. Epithelial cells undergoing EMT are losing cell-cell polarity and adhesion to become migratory, and usually downregulate junctional proteins (such as E-cadherin) whilst modulating their cytoskeleton organization and acquiring mesenchymal features such as vimentin filaments. EMT occurs during development and normal wound repair, while deregulated EMT may occur during cancer metastasis. In addition, it is increasingly recognized that chronic inflammation resulting from repeated injury may also induce persistent EMT, thereby contributing to tissue fibrosis[14]. In the upper airways, downregulation of tight junction-related proteins has been recently described in CRSwNP[15-17], whereas it remains unclear whether this relates to dedifferentiation of the sinonasal epithelium through EMT.

The aim of this study was to evaluate whether the sinonasal epithelium undergoes de-differentiation in CRS and, if so, its relation to lineage

specification and to EMT. Findings were also correlated to airway remodeling and clinical CT score.

MATERIAL AND METHODS

Additional supporting information may be found in the online version of this article.

Subjects and surgical sampling

Patients with CRSsNP (n = 11), CRSwNP (n = 11), AR (n = 10) and controls (n = 13) were recruited at the outpatient clinic of our Department of Otorhinolaryngology. The diagnosis of CRSwNP and CRSsNP was, accordingly to EPOS criteria, based on history, clinical examination, nasal endoscopy and CT scanning[1]. AR was diagnosed based on symptoms and positive skin prick tests and/or serum specific IgE (> 0.35 kU/L) to common aeroallergens, following the ARIA guidelines[2]. Clinical characteristics of the patients are described in Table 1. Controls and AR patients underwent surgery for anatomical obstruction, whilst CRS patients underwent surgery as clinically indicated. All patients were weaned of corticosteroids or antibiotics for at least 3 weeks before surgery, and underwent a preoperative CTscan as part of the clinical work-up, which was graded according to the Lund-Mackay score[18].

Biopsies from inferior turbinates (AR and controls) and from ethmoidal mucosa (CRS) were processed for immunohistochemistry or RT-qPCR.

An informed consent was obtained from each subject, and the study was approved by the local ethical committee (Cliniques Universitaires Saint-Luc, Brussels, Belgium).

	Controls	**CRSsNP**	**CRSwNP**	**AR**
N	13	11	11	10
Sex (M/F)	11/2	7/4	9/2	7/3
Age, years (range)	36.4 (18-62)	37.2 (20-51)	48.2 (28-73)	35.2 (20-48)
History of asthma	1	0	3	1
Atopy	0	0	4	10
Smoking	4 (30%)	4 (36%)	4 (36%)	3 (30%)
CT score	1 (0-2)	12 (7-21)**	17(14-23)***	1 (0-2)

Table 1. Patient characteristics. Patients were assigned to disease groups based on criteria described in Methods, and associated features (asthma, atopic background, cigarette smoking) are stated. Asthma diagnosis was based on the report of typical symptoms and lung function tests, according to GINA[33], and atopy was defined by the presence of skin-prick test or serum IgE responses to at least one common aeroallergen. ** $p<0.01$, *** $p<0.0001$ (Kruskal-Wallis test followed by Dunn post hoc test) for CT scores (Lund-MacKay), as compared to control subjects.

Immunohistochemistry for epithelial differentiation and phenotyping

Five-µm sections of turbinal and ethmoidal biopsies were stained for E-cadherin and Hmw CK as epithelial markers and for vimentin as mesenchymal marker. Lineage markers comprised p63, MUC5AC and ß-tubulin IV for BCs, GCs and CCs, respectively. To quantify the epithelial markers, immunostained sections were scanned using a SCN400 scanner (Leica) and analyzed using TissueIA software (SlidePath). Counting of

vimentin-expressing columnar cells was carried out manually by a blinded observer (CH). Serial stainings for vimentin and CD45 allowed confirming the presence of vimentin-expressing epithelial cells, in addition to intraepithelial leukocytes (Figure E1). Five random epithelial fields were examined per slide, at high magnification (x400). Data are reported for all patients, except when tissue and/or epithelial areas were not sufficiently preserved. BM thickness was measured by multiple point-to-point repeated measurements, as previously described[19].

Quantitative RT-PCR for E-cadherin, CK5, vimentin, ß-tubulin IV, MUC5AC and p63 gene expression

In order to confirm the staining for epithelial markers, mRNA expression levels were quantified in sinonasal tissue and normalized to GAPDH housekeeping gene by using the UPL-probe system (Roche Diagnostics, Basel, Switzerland).

Statistics

Statistical analyses were performed using IBM SPSS Statistics 21.0 (SPSS, Inc, Chicago, USA) and figures were designed using GraphPad Prism 5.00 (GraphPad Software, San Diego, USA). Results are shown as scatter dot plots with mean and standard deviation. Normality was assessed for each data set. For non-parametric data, Kruskal-Wallis test followed by Dunn post hoc test were carried out for multiple comparisons. Mann-Whitney U test was used for the analysis of differences between 2 groups of unpaired data. Normal data were assessed by one-way Anova followed by Tukey post-hoc test for multiple comparisons. Correlation coefficients were

calculated using Spearman's rank method. P-values <0.05 were considered as statistically significant.

RESULTS

Decreased expression of epithelial cadherin and keratins in CRS

To assess the differentiation state of the sinonasal epithelium, tissue sections of control subjects, CRSsNP, CRSwNP and AR patients were first immunostained for E-cadherin and Hmw CK (Figure 1A). E-cadherin was significantly decreased in patients with CRSsNP ($p = 0.03$) and CRSwNP ($p = 0.02$), when compared to controls (Figure 1B). Similarly, Hmw CK expression was decreased in CRSsNP ($p = 0.04$) and CRSwNP ($p = 0.01$), as compared to control subjects (Figure 1C). These results were confirmed by RT-PCR, showing decreased transcription of E-cadherin in CRSwNP and CRSsNP, while CK5 mRNA (a major Hmw CK, preferentially expressed by basal cells) was also significantly decreased in CRSwNP (Figure 1D).

Number of ciliated and goblet cells are not significantly changed in CRS

To address whether these epithelial changes related to changes in epithelial specification, numbers of CCs, GCs and BCs were evaluated in the CRS sinonasal epithelium by immunohistochemistry and RT-qPCR (Figure 2A). No significant changes were observed for β-tubulin IV + CCs and MUC5AC+ GCs, and this was confirmed at the mRNA level for these markers (Figure 2B-C). The number of p63+ BCs was decreased in

CRSsNP, as compared to controls ($p = 0.008$) (Figure 2B), while this change was not observed at the mRNA level (Figure 2C).

These data suggested that the epithelial de-differentiation may not be related to changes in the lineage specification of the upper airway epithelium.

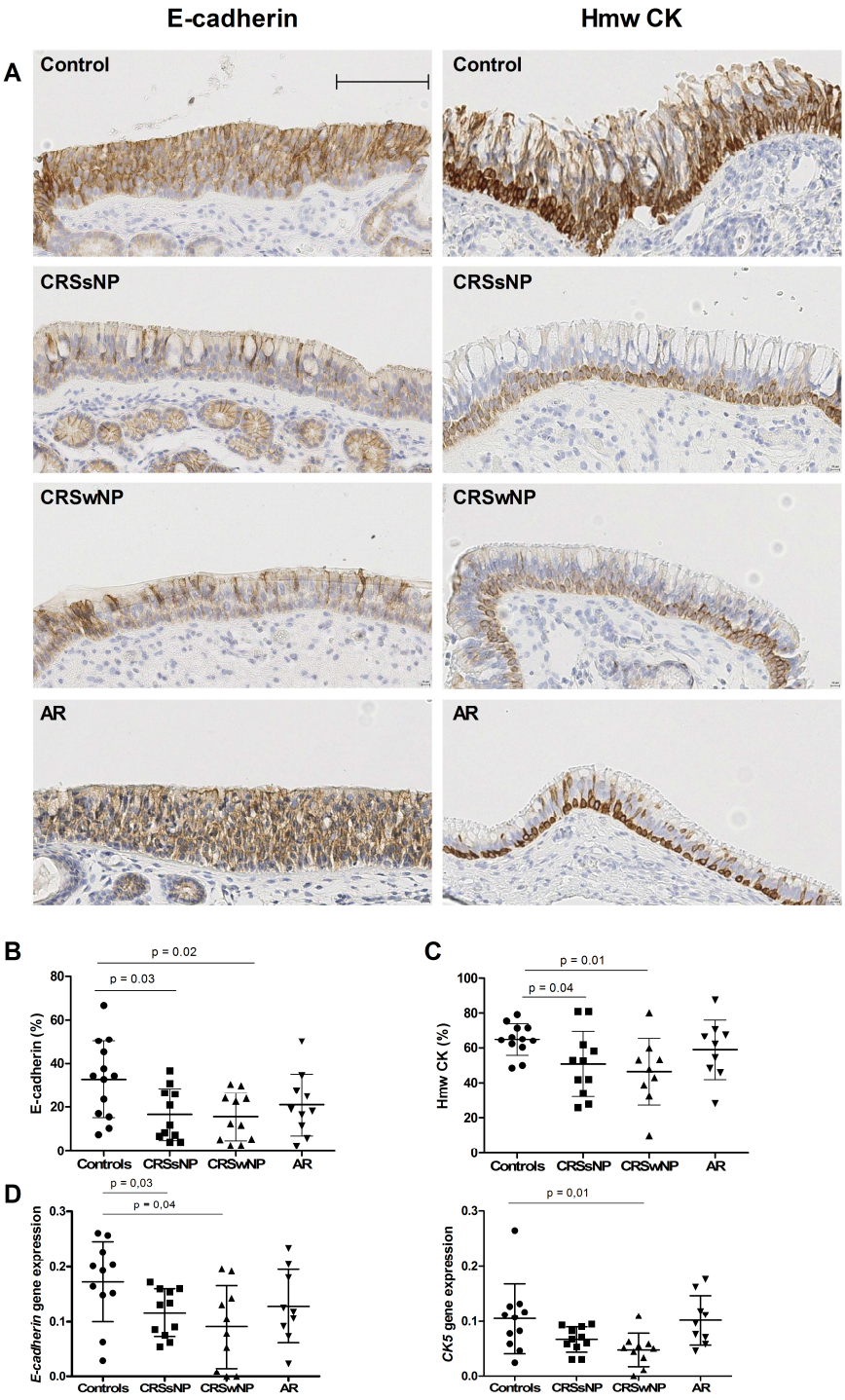
E-cadherin
Hmw CK
A
Control
Control
CRSsNP
CRSsNP
CRSwNP
CRSwNP
AR
AR
B
p = 0.02
p = 0.03
E-cadherin (%)
Controls
CRSsNP
CRSwNP
AR
C
p = 0.01
p = 0.04
Hmw CK (%)
Controls
CRSsNP
CRSwNP
AR
D
p = 0,03
p = 0,04
E-cadherin gene expression
Controls
CRSsNP
CRSwNP
AR
p = 0,01
CK5 gene expression
Controls
CRSsNP
CRSwNP
AR

Figure 1: Immunohistochemistry for epithelial markers in sinonasal tissue.

A. Immunostaining for E-cadherin (left) and Hmw CK (CK50) (right) in sinonasal epithelium. Representative images from each group are shown (scale bar, 100µm).
B. Quantification of E-cadherin staining using TissueIA software (SlidePath) (n= 45).
C. Quantification of the Hmw CK staining using TissueIA software (SlidePath) (n=41).
D. E-cadherine (left) and CK5 (right) mRNA expression in sinonasal mucosa from patients with CRSwNP, CRSsNP and AR, as compared to controls, and normalized to GAPDH housekeeping gene.

Increased numbers of vimentin-expressing epithelial cells in CRS

We then evaluated whether epithelial de-differentiation could relate to the acquisition of a mesenchymal phenotype, by assessing the number of vimentin positive epithelial cells in the sinonasal epithelium of our patients. The number of vimentin expressing epithelial cells was significantly increased in CRSsNP ($p=0.001$) and CRSwNP ($p<0.0001$), as compared with controls (Figure 3A-C). In addition, increased vimentin-expressing epithelial cell numbers correlated with the radiological (Lund-McKay) score ($r = 0.59$, $p<0.0001$) (Figure E2-C). It was also correlated with the number of eosinophils ($r = 0.5$, $p =0.004$) assessed in our previous study of the same series of patients[20] (figure E2-A).

Increased subepithelial fibrosis in CRS

We then evaluated whether this EMT feature was associated with subepithelial fibrosis, by measuring the BM thickness in the same epithelial areas (Figure 3B). We observed increased BM thickness in CRSsNP ($p = 0.04$) and CRSwNP ($p = 0.01$) (Figure 3D). In addition, BM thickening

correlated with the CT score (r = 0.48, p = 0.003) (Figure E2-D) and with vimentin-expressing epithelial cells (r = 0.54, p = 0.0001) (Figure 3F). Moreover, the BM thickness correlated with tissue eosinophilia (r=0.36, p=0.04) (Figure E2-B).

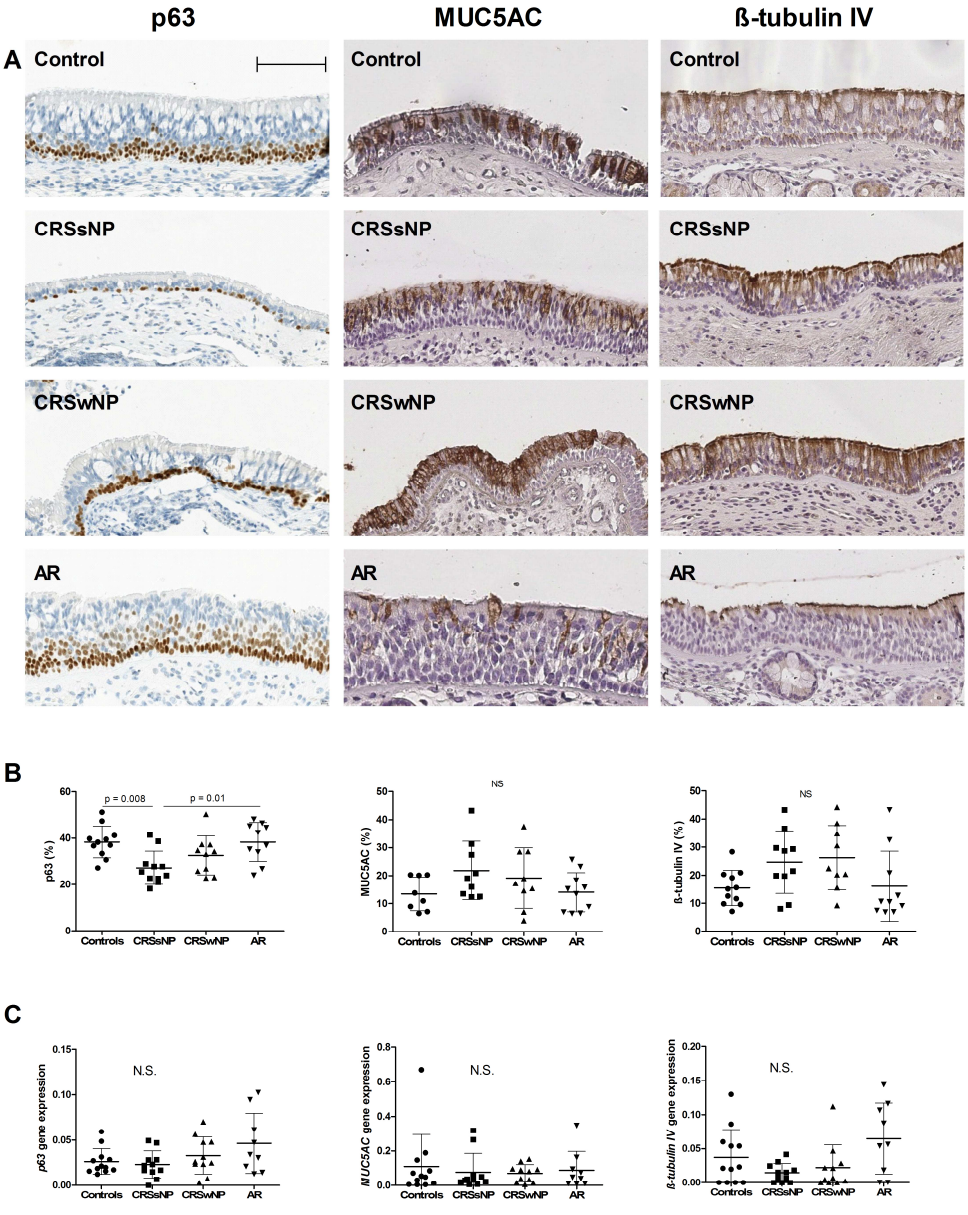

Figure 2: Immunohistochemistry for different cell types in sinonasal tissue.

A. Immunostaining for p63 (basal cells, left), MUC5AC (goblet cells, middle) and ß-tubulin IV (ciliated cells, right). Representative images from each group are shown (scale bar, 100µm).
B. Quantification of p63 (n=42), MUC5AC (n = 36) and ß-tubulin IV (n=40) staining in sinonasal epithelium, expressed as percentage of positive cells (p63) and percentage of stained area (MUC5AC and ß-tubulin IV).
C. p63, MUC5AC and ß-tubulin IV mRNA expression in sinonasal mucosa from patients with CRSwNP, CRSsNP and AR, as compared to controls, and normalized to GAPDH housekeeping gene.

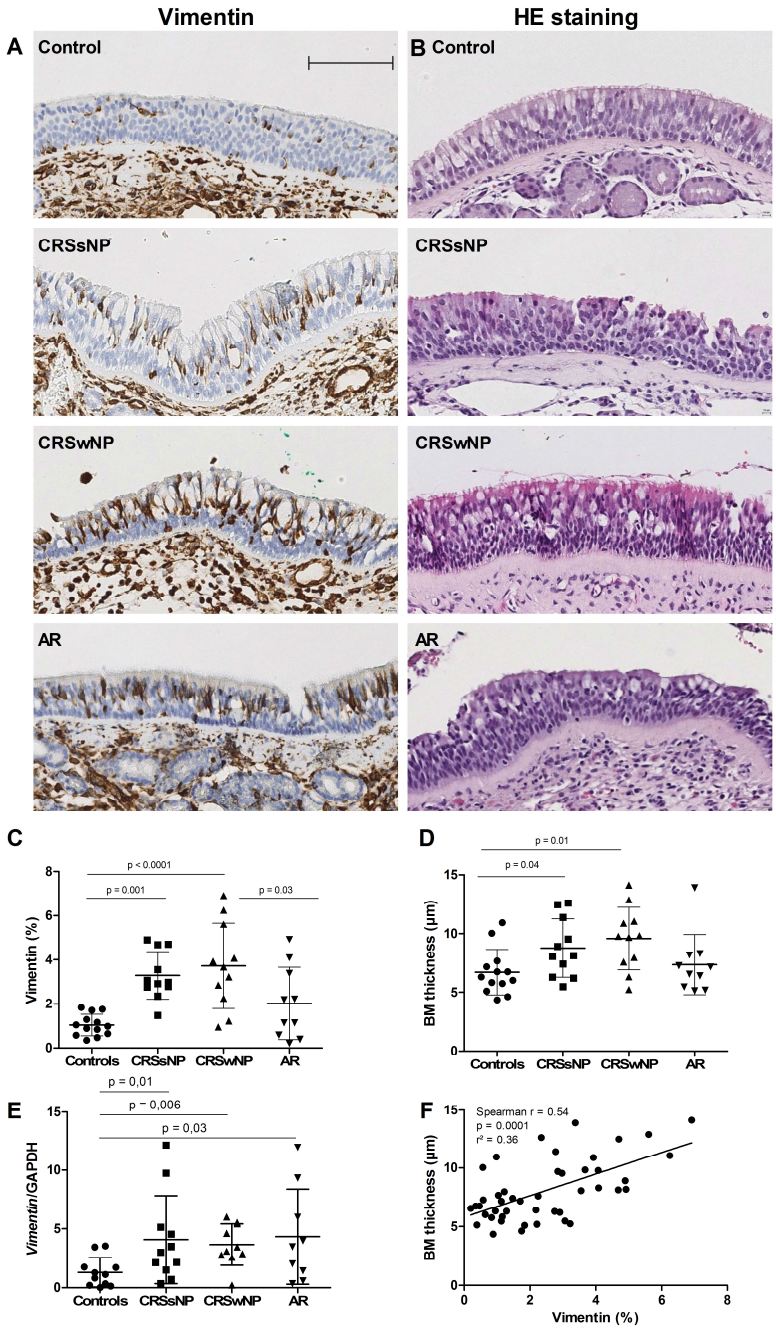
Vimentin
HE staining
A
Control
CRSsNP
CRSwNP
AR
B Control
CRSsNP
CRSwNP
AR
C
p < 0.0001
p = 0.001
p = 0.03
Vimentin (%)
Controls
CRSsNP
CRSwNP
AR
D
p = 0.01
p = 0.04
BM thickness (µm)
Controls
CRSsNP
CRSwNP
AR
E
p = 0,01
p = 0,006
p = 0,03
Vimentin/GAPDH
Controls
CRSsNP
CRSwNP
AR
F
Spearman r = 0.54
p = 0.0001
r² = 0.36
BM thickness (µm)
Vimentin (%)

Figure 3: Vimentin and fibrosis in sinonasal tissue.

A. Immunostaining for vimentin in sinonasal epithelium. Representative images from each group are shown (scale bar, 100µm) (n=45).
B. Evaluation of BM thickness; representative hematoxylin-and-eosin stained images from each group are shown.
C. Quantification of vimentin staining in sinonasal epithelium (n=45), expressed as percentage of positive cells.
D. Measurement of the BM height using TissueIA software (SlidePath) (n=45).
E. Vimentin mRNA expression in sinonasal mucosa from patients with CRSwNP, CRSsNP and AR, as compared to controls, and normalized to GAPDH housekeeping gene.
F. Correlation of vimentin expression to BM thickness.

Discussion

This study shows in a large series of well-characterized patients that the sinonasal epithelium undergoes de-differentiation in CRS, which does not relate to changes in lineage differentiation (CCs/GCs), but to mesenchymal transition. In addition, percentage of vimentin-expressing epithelial cells correlates with subepithelial fibrosis evidenced by BM thickness and with the disease severity (CT scoring), suggesting a role in disease pathogenesis.
E-cadherin is a transmembrane glycoprotein that establishes and stabilizes cellular contacts in adherens junctions between adjacent epithelial cells. E-cadherin is involved in the control of several aspects of epithelial homeostasis, including morphological differentiation, cell proliferation and cell motility, notably during wound healing and inflammation[21]. A defective epithelial barrier has been recently described in patients with CRSwNP, along with a decreased expression of tight junction proteins, such as claudin-4, occludin, zonula occludens-1[15, 16]. We confirm a defect in

junctional complexes, by showing decreased expression of adherens junction-associated E-cadherin in CRSwNP. In addition, this epithelial defect was also observed in CRSsNP, suggesting that this feature may occur in different phenotypes of chronic sinusal disorders.

Our data suggest that EMT occurs in the upper airways of patients with CRS. In the lung, EMT has been shown in lung fibrosis[14] and obliterans bronchiolitis[22] and is debated in COPD[23] and asthma[24]. During EMT, epithelial cells undergo phenotypic transition to mesenchymal cells, acquiring the intermediate filament vimentin in their cytoskeleton, subsequently become spindle shaped, and exhibiting front-to-back, leading-edge polarity[13]. EMT has not been specifically studied in chronic sinonasal diseases. One previous study showed the presence of vimentin+ epithelial cells in both normal nasal mucosa and CRS, and suggested that these cells may arise from EMT[25]. A recent paper showed a trend to a higher, but not significantly, number of vimentin+ cells in the turbinate part of nasal polyps, as compared to normal mucosa[16]. Our study shows that the vimentin expression is increased in the epithelium from patients with CRSwNP and CRSsNP, as well as to a lesser extent (only at the transcriptional level) with AR. BM thickening has been described in asthma[26], COPD[27], and CRS[28]. Accordingly, we found a thickening of the BM in CRSsNP and CRSwNP, while clefts identified within the BM from COPD patients[27] were not observed (not shown). The low number of vimentin-expressing epithelial cells may indicate that EMT selectively concerns certain cells and, although quantitatively limited, may reflect a profound reprogramming of the upper airway epithelium in CRS also characterized by the loss of junctional proteins (Figure 4). In addition, epithelial vimentin expression correlated

with BM thickening, and both vimentin and BM thickness correlated with the radiological score, suggesting that EMT correlates to disease severity in CRS and could contribute to its pathogenesis.

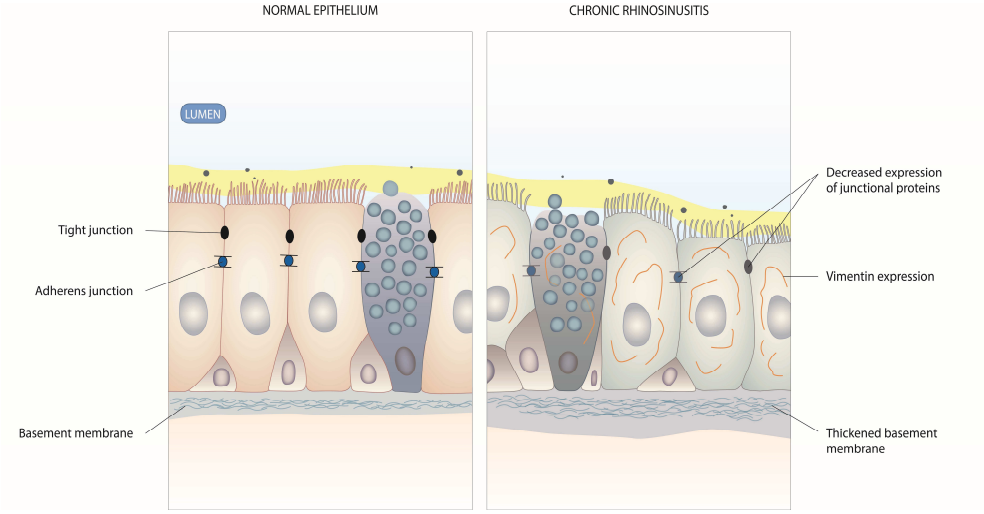

Figure 4: Epithelial-to-mesenchymal transition in chronic rhinosinusitis
De-differentiation and EMT features are observed in CRSwNP and CRSsNP, characterized by decreased expression of junctional proteins associated with tight and adherens junctions, increase of intracellular vimentin filaments, and thickening of the basement membrane.

In contrast to these differentiation features, no significant change in lineage differentiation was observed in CRS when assessing the two main differentiated cell types, namely GCs and CCs[5]. BCs are relatively undifferentiated cells, considered as a multipotent progenitor population and a common feature of stratified and pseudostratified epithelia throughout the body[6]. During normal epithelial turnover and repair, they progressively differentiate into other cell types, such as GCs or CCs, reconstituting a functional respiratory epithelium[6]. An increase of BCs has been reported in

CRSwNP[12]. In contrast, we observed a decrease in p63+ BCs in patients with CRSsNP, while p63 mRNA expression was not affected, and a decrease in mRNA expression of CK5 (a CK preferentially expressed by BCs, along CK14) in CRSwNP. It remains thus unclear whether BC numbers are contributing to epithelial changes. In addition, whereas mucus-secreting GCs show varying density in the nasal mucosa (with a peak of 10.000 cells/mm² in the maxillary sinus[29]) and changes in patterns of mucin gene expression[11, 29], studies showed contradictory results with either a decrease[11] or an increase[10] of MUC5AC expression in CRSwNP. In our study, no significant change in the percentage of MUC5AC+ epithelial cells was observed. Similarly, no change was observed for ß-tubulin IV+ CCs, considered as terminally differentiated columnar cells, both at protein and mRNA levels. These data suggest that, although the number of BCs is probably highly variable according to the turnover of the upper airway epithelium and may be affected in CRS, the differentiation programming into the two main cell types (CCs and GCs) is preserved in CRS.

In contrast, de-differentiation and EMT features were observed both in CRSwNP and CRSsNP. As these two disorders generally differ in terms of immune profile, this observation may indicate that these changes could occur independently of the nature of the inflammatory reaction. Similarly, EMT has been suggested in the bronchial epithelium both in asthma and COPD[23, 24]. However, we observed that vimentin expression correlated with tissue eosinophils. It is known that damage to the epithelium closely correlates with the degree of eosinophil infiltration, and that eosinophil products can cause epithelial damage and submucosal oedema[30]. Activated

eosinophils contribute to CRSwNP through the release of major basic protein in the production of cytokines[31]. It could therefore be speculated, although several pathways could be involved, that eosinophils may contribute to epithelial de-differentiation and EMT. Alternatively, epithelial dysfunction could favour eosinophilia through several mechanisms such as impaired exclusion of proeosinophilic superantigens from *Staphylococcus Aureus*[32]. We recently reported decreased levels of IgA antibodies to *Staphylococcus Aureus* enterotoxin B in the nasal fluid from patients with CRSwNP[20], as the putative result of impaired pIgR expression in the sinonasal epithelium, which closely relates to epithelial differentiation (*Gohy AJRCCM 2014* provisionally accepted on 30 July 2014*).*

It is also very likely that EMT *per se* affects the epithelial barrier against pathogens, by defects in junctional complexes and in epithelial secretion of antimicrobial factors.

In summary, our data indicate for the first time that epithelial de-differentiation towards a more mesenchymal phenotype occurs in the upper airways of patients with CRSsNP and CRSwNP, independently of lineage differentiation into ciliated and goblet cells. This epithelial reprogramming correlates with subepithelial fibrosis and with disease severity. These findings also highlight common mechanisms of epithelial dysfunction between upper and lower airways, occurring during chronic inflammatory diseases. Future studies should address the underlying mechanisms, which could represent future therapeutic targets in CRS.

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16. Meng J, Zhou P, Liu Y, et al. The development of nasal polyp disease involves early nasal mucosal inflammation and remodelling. *PloS one* 2013; **8**(12): e82373.

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Supporting Information

Methods

Immunohistochemistry

After surgical removal, the turbinal and ethmoidal biopsies were immediately immersed in 4% formaldehyde in phosphate-buffered saline at pH 7.4 for at least 24 h. Samples for immunohistology were embedded in paraffin. Serial sections of 5 μm thickness were cut from paraffin blocks, spread on Superfrost Plus glass slides, and dried at 40° C for at least 24 h.

The slides were then processed for immunostaining, each step of the procedure being followed by washing with Tris-buffered saline (TBS) with 0.05% Tween 20 (Sigma-Aldrich, Saint-Louis, USA) (pH 7.4) After deparaffinization and rehydration of the specimen, endogenous peroxidases were inhibited by incubation in 0.3% (vol/vol) H_2O_2 in water for 30 min. Antigen retrieval was performed in a citrate buffer during 75 minutes at 98°C. A blocking step was then performed by incubating slides in 5% (wt/vol) BSA in TBS-Tween for 30 min and was followed by a 10 min incubation with avidin and biotin 0.001% to block endogeneous biotins. Slides were then incubated overnight at 4°C with primary antibody diluted in TBS-Tween containing 1% BSA followed by anti-mouse IgG (whole molecule)-biotin (Sigma-Aldrich, Saint-Louis, USA) applied in 2% (wt/vol) defatted dry milk for 30 min at RT.

The reaction was amplified with streptavidin horseradish peroxidase conjugate (HRP) (BD Bioscience, San Jose, USA) in TBS-Tween

containing 1% BSA for 30 min, and revealed with Ultra View Universal 3, 3-diaminobenzidine detection kit (Roche, Basel, Switzerland) according to manufacturer's instructions. Slides were counterstained with Mayer's hematoxylin and mounted with Safemount medium.
Immunostaining was performed following this protocol for anti- human E-cadherin (clone NCH-38, DAKO, Glostrup, Denmark), anti-human Hmw CK (CK50, clone 34bE12, DAKO, Glostrup, Denmark), anti- human vimentin (clone V9, DAKO, Glostrup, Denmark), anti- human CD45 (clones2B11+PD7/26, DAKO, Glostrup, Denmark), anti-human p63 (clone 4A4 + Y4A3, ThermoScience), anti-human β-tubulin IV (clone ONS.1A6, Sigma-Aldrich, Saint-Louis, USA), anti-human MUC5AC (clone CLH2, Leica, UK) antibodies. Control sections were treated as appropriate with normal mouse IgG isotype at same dilution.

Slides were scanned with a SCN400 scanner (Leica), calibrated for brightness and white balance. Stained areas over threshold were quantified within manually delineated epithelium, using TissueIA software (SlidePath). The threshold was established according to negative and positive controls. The same threshold was applied for all slides.

Five random epithelial fields (x400 magnification) from each sample were analyzed.

Results for E-cadherin, Hmw CK (CK50), MUC5AC and ß-tubulin IV were calculated and expressed as the percentage of positive/stained area within the epithelium, using TissueIA software (SlidePath). P63 positive cells were calculated and expressed as percentage of stained cells per total cells in the epithelial field, using the same software. Vimentin positive cells were

counted manually by a single blinded observer, reported to the total number of epithelial cells, and expressed as percentage of stained cells per epithelial field. A serial staining of vimentin and CD45 allowed us to exclude inflammatory cells that could also express mesenchymal markers. These inflammatory cells, even if positively stained, were not taken in account in the counting of vimentin positive cells.

Quantification of the thickening of reticular basement membrane on vimentin staining pictures was performed by multiple point-to-point repeated measurements at regular intervals of 20µm on the same fields as for vimentin quantification (x400 magnification).

Quantitative RT-PCR for ß-tubulin IV, MUC5AC and p63

Total RNA was isolated using the Rneasy® Plus Mini kit (Qiagen). 500 ng of total RNA was reverse-transcribed into cDNA with RevertAidTM Reverse transcriptase kit (Fermentas, Vilnius, Lithuania). Vimentin, E-cadherin, cytokeratin 5 (CK5), p63, ß-tubulin IV and MUC5AC expression were quantified and normalized to GAPDH by qPCR using the UPL-probe system (Roche Diagnostics, Basel, Switzerland). qPCR reactions were performed using the "Fast Start Universal Probe Master" kit as recommended by the manufacturer, in the LightCycler 480 Instrument (Roche Diagnostics). The reverse and forward primers (Eurogentec, Seraing, Belgium) for vimentin, E-cadherin, CK5, p63, ß-tubulin IV, MUC5AC and GAPDH were designed as follows: vimentin primers (Forward: GCG TGA CGT ACG TCA GCA ATA TGA, Rev: GTT CCA

GGG ACT CAT TGG TTC CTT), E-cadherin primers (For: GAC ACA TTT ATG GAA CAG AAA ATA ACA, Rev: AGT GGA AAT GGC ACC AGT GT), CK5 primers (For: TTC ATG AAG ATG TTC TTT GAT GC, Rev: AGG TTG CGG TTG TTG TCC), p63 primers (For: CGC CAT GCC TGT CTA CAA, Rev: TGA CTA GGA GGG GCA ATC T), ß-tubulin IV primers (For: CCG GAC AAC TTC GTG TTT G, Rev: ACA GCG TCC ACC AGC TCT), MUC5AC primers (For: CAC GTC CCC TTC AAT ATC CA, Rev: GGC CCA GGT CTC ACC TTT) and GAPDH primers (For: ACC AGG TGG TCT CCT CTG AC, Rev: TGC TGT AGC CAA ATT CGT TG). Each sample was quantified using a standard curve made of serial dilutions of a batch of human nasal polyp cDNA (made with the "Transcriptor First Strand cDNA Synthesis" kit-Roche).

Additional figures

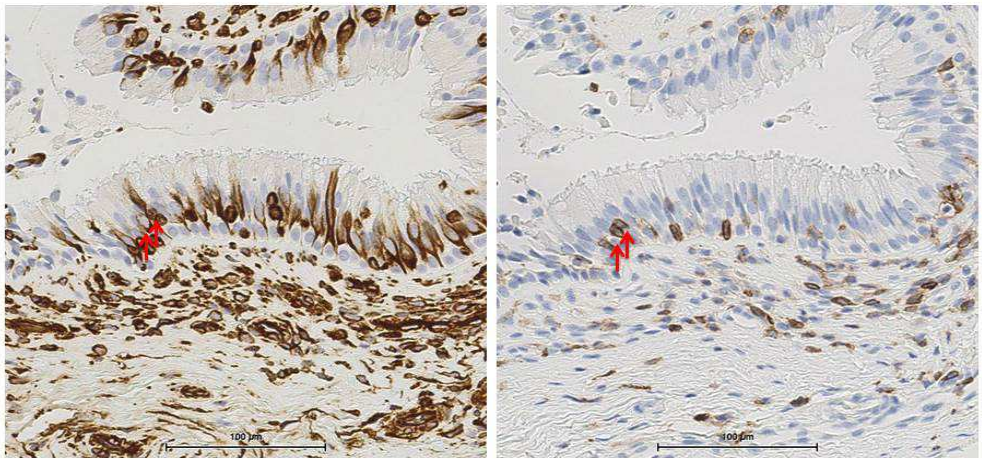


Figure E1: Serial immunostaining (5µm between sections) for vimentin (left) and CD45 (right). Red arrows show intraepithelial leukocytes that were not taken in account for vimentin quantification. Scale bar, 100µm.

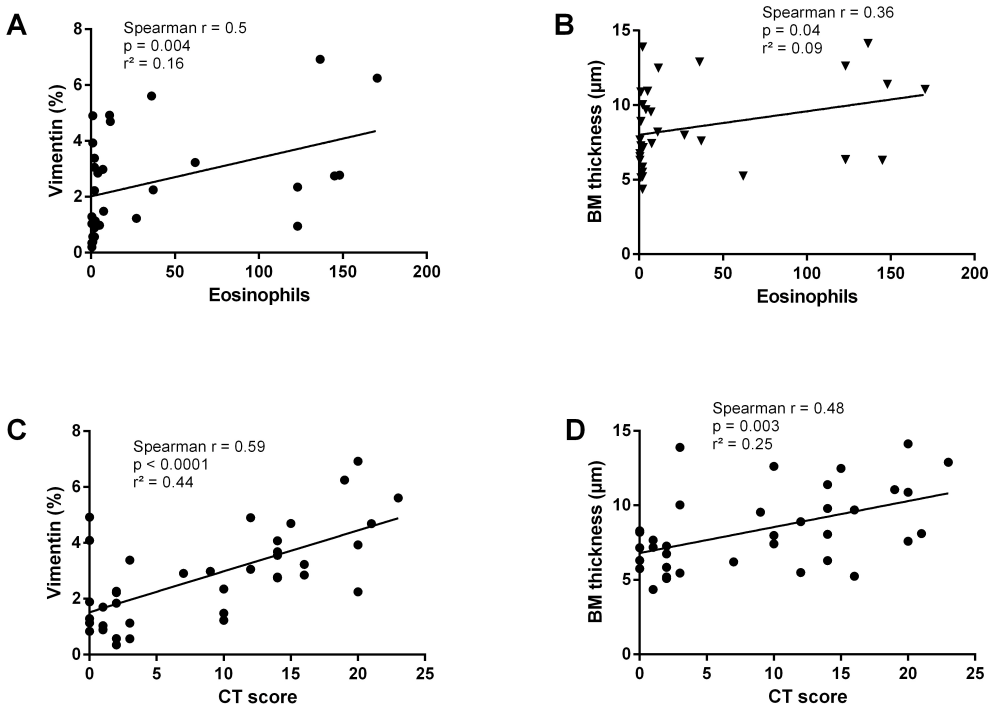


Figure E2: Correlations between epithelial vimentin expression, eosinophils, subepithelial fibrosis and clinical CT score of sinusitis.

A. Correlation between vimentin-expressing epithelial cells and eosinophil numbers.

B. Correlation between BM thickness and eosinophil numbers.

C. Correlation between vimentin-expressing epithelial cells and Lund-McKay CT score.

D. Correlation of BM thickness and Lund-McKay CT score.

CHAPTER 5: COULD STEROIDS AND IMMUNOGLOBULIN E- OR IL-5-TARGETED BIOTHERAPIES RESTORE SECRETORY IGA RESPONSES IN NASAL POLYPOSIS?

ASSESSMENT OF S-IGA ANTIBODIES IN CRSWNP PATIENTS TREATED BY METHYLPREDNISOLONE, DOXYCYCLINE, MEPOLIZUMAB OR OMALIZUMAB, VS PLACEBO

Introduction

As we observed that pIgR expression is decreased in CRSwNP, including specific S-IgA antibodies to Stapylococcus Aureus along subepithelial IgA accumulation due to impaired pIgR-mediated transport, we thought to evaluate the effects of medical treatments of CRSwNP on IgA production in order to address the possibility that local inflammation could condition the airway epithelium for pIgR downregulation. This study was carried out in collaboration with Prof. Ph. Gevaert (Upper Airways Research Laboratory, UZ Gent) who kindly provided nasal and serum samples from different controlled trials performed in their department.
IgA was assessed by ELISA in nasal secretions from CRSwNP patients treated by Doxycycline, Methylprednisolone, Mepolizumab or Omalizumab vs placebo.

We present here the results of these investigations.

Material and Methods

ELISA for SC and IgA subclasses

Total IgA, SC, IgA1, IgA2 and specific IgA to Staphyloccoccus Aureus enterotoxin B concentrations were determined in nasal secretions by a specific enzyme-linked immunosorbent assay (ELISA). For SC, IgA1 and IgA2, 96-well microplates were coated with 1 μg/ml affinity-purified goat anti-SC antibody (developed in our laboratory, recognizing both soluble SC

and membrane pIgR/SC) in 0.1 M carbonate-bicarbonate buffer, pH 9.6, overnight at 4°C. After washings with 1/1.000 vol/vol Tween 20-PBS (PBST) and blockade with 1% wt/vol BSA in PBST for 1h at 37°C, diluted samples and serial dilutions of purified human soluble SC/IgA1/IgA2 were incubated for 2 h at 37°C. After washings with PBST, plates were then incubated with biotinylated goat anti-SC/ biotinylated mouse anti-IgA1/ biotinylated mouse anti-IgA2 for 2 h and washed in TBST.

A volume of 100 μl streptavidin–polyHRP in a 1:10.000 dilution in HPE buffer was added to each well and incubated for 30 min at room temperature. The plates were washed three times using PBST, and 100 μl of (3,5,3′,5′)-Tetramethylbenzidine substrate was added to each well. After incubation, the reaction was stopped by the addition of 100 μl of 2 M H_2SO_4. The absorbance was measured at 450 nm.

Total IgA concentration was determined by the same method (coating with 1 μg/ml affinity-purified goat anti-SC antibody, blockade with 1% wt/vol BSA in PBST, diluted samples and serial dilutions of purified total IgA) except for the secondary antibody which was anti-human IgA (α-chain specific)−peroxidase antibody produced in goat. As it was peroxidase-conjugated, use of streptavidin–polyHRP was not necessary.

To assess specific IgA to pneumococcal anticapsular antigens and Staphylococcus aureus enterotoxin B, the same method was used, adapted from a previous report.[1] Microplates were coated with the whole 23-valent pneumococcal polysaccharide vaccine (Sanofi-Pasteur MSD) or with Staphylococcal Enterotoxin B from Staphylococcus aureus (Sigma-Aldrich).

The detection antibody was HRP-conjugated goat anti-human IgA (α-chain specific). Results were expressed as arbitrary units, by reference to serial dilutions of a highly positive sample (OD given by 1:2 dilution set as 1 unit, and corrected to the dilution factor).

Values obtained by ELISA were corrected with a dilution factor.

Patients and study design

All measures were realized on serum and nasal fluid samples collected during 3 different studies conducted by the Department of Otorhinolaryngology of the University Hospital in Ghent, Belgium[2-4]. Nasal fluid was collected as previously described (Watelet et al.)[5].

Subjects from Doxycycline-Methylprednisolone study (adapted from Van Zele et al.[2])

Fourty-six healthy patients with recurrent CRSwNP after surgery or massive CRSwNP were included. The use of systemic or local corticosteroids or antibiotics was not allowed during the study; if necessary, patients were permitted to use nasal corticosteroids as rescue medication 2 months after dosing with the study medication.

Patients were randomly assigned to 3 groups : groups were given either oral methylprednisolone (32 mg/d on days 1-5; 16 mg/d on days 6-10; and 8 mg/d on days 11-20, 14 subjects), oral doxycycline (200 mg on day 1, 100 mg/d on days 2-20, 15 subjects), or placebo (lactose, 17 subjects) in unlabeled capsules. Follow-up visits were scheduled for 1, 2, 4, 8, and 12 weeks after dosing.

IgA measurements were realized on serum and nasal fluids samples collected at visit 1, 3 (after 4 weeks) and 4 (after 8 weeks).

Subjects from Mepolizumab study (adapted from Gevaert et al.[3])

Twenty-seven subjects with primary CRSwNP (based on EPOS criteria) or CRSwNP recurrent after surgery were included. The inclusion criteria specified that subjects must have had failure of standard care for CRSwNP. Use of systemic corticosteroids and surgical intervention was not allowed from 1 month before treatment until the end of the study, and subjects were not permitted to use nasal corticosteroids, nasal antihistamines, nasal atropine, nasal cromolyn, nasal saline, or antibiotic treatment for 2 months after first dosing. A randomized, double blind, placebo-controlled study of mepolizumab was performed in patients with CRSwNP. After signing the informed consent form and a 4- to 12-week run-in period, subjects were randomized to receive 2 single intravenous injections (28 days apart) of 750 mg of mepolizumab (18 subjects) or placebo (9 subjects). Follow-up visits were scheduled 1, 4, 8, 12, 24, 36, and 48 weeks after first dosing. During the follow-up visit after 4 weeks, the second injection of mepolizumab was administered. All randomized patients were included in the analysis. The study was double blind up to 48 weeks.

IgA measurements were realised on serum and nasal fluids samples collected at visit 1, 3 (after 8 weeks) and 4 (after 12 weeks).

Subjects from Omalizumab study (adapted from Gevaert et al.[4])

Twenty-two subjects with CRSwNP (EPOS guidelines) and asthma (GINA guidelines) were included in the study. Their serum IgE levels were comprised between 30 and 700 kU/mL. All patients underwent skin prick tests and both allergic and non-allergic patients were included in the study.

After a 2-week run-in period, subjects were randomized on a 2:1 basis to receive subcutaneous treatment with anti-IgE (14 subjects) or placebo (8 subjects). Both the investigator and the subject were blind to study treatment. The dose (in milligrams) and dosing frequency (every 2 weeks/8 injections in total or every month/4 injections in total) of omalizumab (Xolair; Novartis, Basel, Switzerland) were based on total serum IgE levels (in international units per milliliter) and body weight (in kilograms), with a maximum dose of 375 mg. After screening, 10 visits were scheduled every 2 weeks over 20 weeks.

IgA measurements were realized on serum and nasal fluids samples collected at visit 1, 4 and 6.

Results

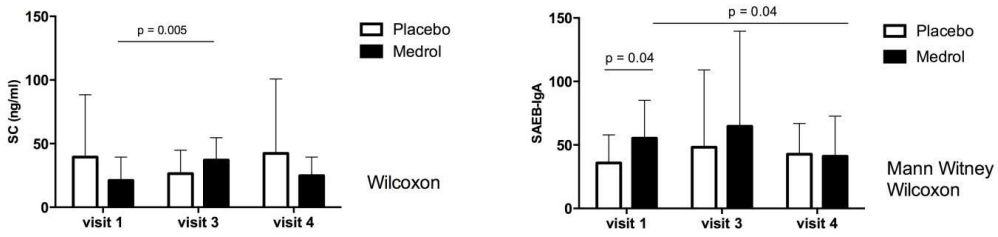
In the three different studies, all previous mentioned IgA subclasses and SC were measured, however, in order to simplify the following chapter, only significant results are shown.

1. **Doxycycline-Methylprednisolone study**

a) **Nasal secretions**

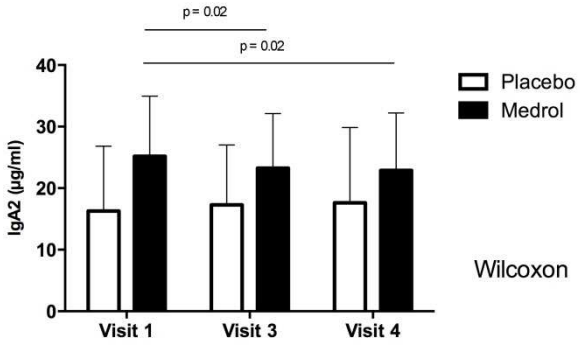
SC is increased in nasal fluid from CRSwNP patients one week after methyprednisolone treatment ($p = 0.005$), but this increase did not persist on the subsequent visit.

Conversely, the level of SAEB-specific IgA was decreased 4 weeks after methyprednisolone treatment.



b) Serum

We observed no significant changes in IgA levels in the methyprednisolone group (vs placebo), except a minor decrease of IgA2 between visit 1 and visit 3 and between visit 1 and visit 4.

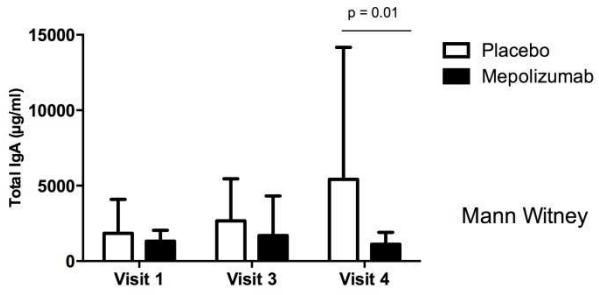


Patients treated with doxycycline (as compared to placebo) showed no significant changes in IgA levels upon visit 3 and visit 4, neither in nasal secretions nor in serum.

2. Mepolizumab study

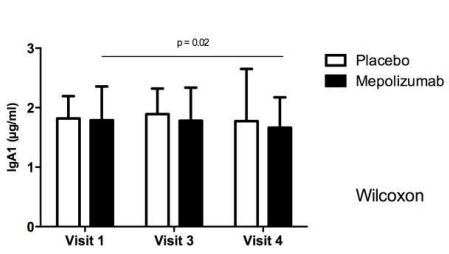
a) Nasal secretions

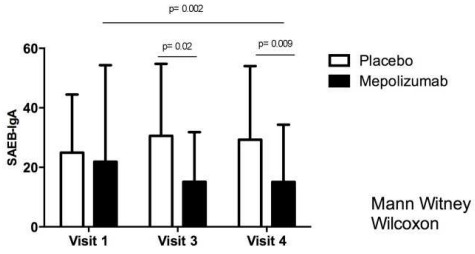
No significant effect of mepolizumab was observed vs placebo, except a decrease in total IgA after 4 weeks, which was associated with a large dispersion of values in the placebo group.



b) Serum

There was no change in serum IgA levels upon mepolizumab treatment vs placebo, while a very modest decrease was observed in IgA1 levels over time (4 weeks vs 1 week), as also seen for SEB-specific IgA.

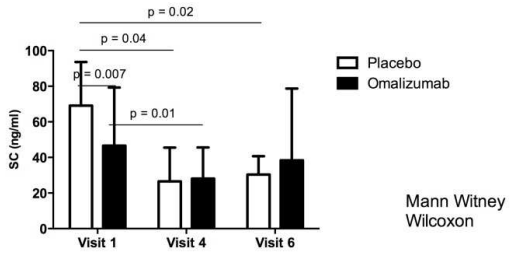


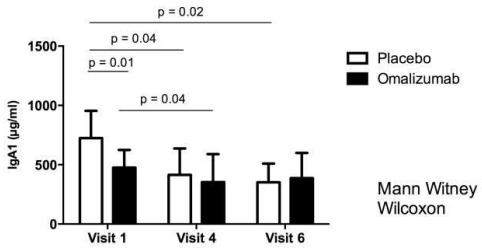


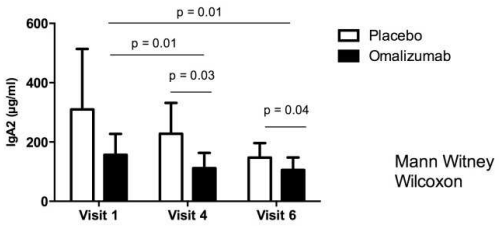
3. Omalizumab study

a) Nasal secretions

We observed reduced IgA1, IgA2 and SC production in nasal secretions from patients treated by omalizumab at visit 4 and 6.







b) Serum

No significant differences were observed among IgA levels in the serum of patients treated by omalizumab as compared to placebo.

Conclusions

The main information about these results is thus that secretory component is increased in nasal secretions of CRSwNP patients treated by methylprednisolone, after one week of treatment, but that this effect does not persist with time. This increase in SC could be due to a higher turnover of pIgR and/or a higher expression of the receptor, influenced by methylprednisolone, as we showed previously that pIgR expression was downregulated in CRswNP patients. This unknown effect of methylprednisolone could contribute to the decrease of nasal symptoms observed in CRSwNP patients during methylprednisolone treatment, and to the rebound effect after the end of the treatment.

However, these results must be confirmed, especially by prospective studies, where the impact of methylprednisolone could be studied on IgA and SC levels, but also on pIgR expression, and correlated to clinical symptoms.

Other changes in IgA levels were either very modest and/or of uncertain clinical relevance. This could be due, in part, to the fact that samples had to be highly diluted to perform the ELISA assays, as IgA levels in nasal secretions were very high. As a consequence, these results need to be confirmed by other techniques, such as immuno-nephelometry or ImmunoCap, and correlated to the local expression of pIgR or IgA levels in biopsies or tissue homogenates.

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CHAPTER 6: GENERAL CONCLUSIONS

General conclusions

The main objective of this thesis was to investigate the secretory IgA system in chronic upper airway diseases, and its link with eosinophilic inflammation and epithelial changes.

In the first study, we confirmed that, as described previously in COPD[1], pIgR expression is reduced in CRSwNP and AR. This reduced expression results in reduced secretion of SC and specific IgA antibodies to SAEB in nasal fluid. IgA subclasses, however, were not significantly affected in nasal fluid, but accumulated at subepithelial level. We hypothesized that the increased IgA levels described in this work and, earlier, by Van Zele et al.[2] were thus a reflection of an aborted IgA transport into secretions due to impaired epithelial pIgR expression. Interestingly, similar results were published simultaneously by Hulse et al.[3], confirming a reduced expression of pIgR in CRSwNP, with increased levels of IgA in tissue homogenates, but without significantly increased IgA levels in nasal lavage fluid. Contrarily to us, they hypothesized that these higher IgA levels in tissue were due to an increased IgA production, with a pIgR system unable to upregulate its function. In our views, IgA accumulation rather relates to impaired epithelial transport, as indices of active synthesis of IgA1 and IgA2 were unchanged.

Moreover, we showed that pIgR positively correlates to IL-12 expression as Th1 signature, but, most importantly, that pIgR downregulation is closely related to Th2-type eosinophilic inflammation. It has been postulated many years ago that IgA may be the principal Ig mediating eosinophil effector function at mucosal surfaces[4]. High quantities of IgA in the tissue might

thus further exacerbate inflammation through degranulation of eosinophils, among other pathways.

In the second study, we assessed epithelial changes in terms of lineage and terminal differentiation. We showed a decrease of terminal differentiation marker expression (E-caderin and Hmw CK) as well as an increase in fibrosis (vimentin expression and basal membrane thickening) in CRSwNP and CRSsNP, while the number of ciliated and goblet cells remained unaffected. This decrease in E-cadherin and cytokeratins, associated with an increase in vimentin, could be considered as the first signs of EMT in CRSwNP and CRSsNP. A reduction of E-cadherin and/or junctional proteins has been described previously in chronic upper airway diseases [5-8], but was not asssociated to a significant increase in vimentin.

It is interesting that, as in asthma and COPD[9, 10], EMT is present in CRSwNP and CRSsNP. This observation highlights two important points: it reinforces the already known link between upper and lower airways, and it might suggest that EMT is not directly related to a specific inflammatory profile. The increase in vimentin however, correlates to eosinophilic inflammation, independently of the disease. As epithelial damage is linked to eosinophilic infiltration[11], we suggested that eosinophils might play a role in epithelial de-differentiation and EMT. It could also be hypothesized that EMT itself could induce a compromised mucosal epithelial barrier, as part of the epithelial cells differentiate into mesenchymal cells, reducing thereby their capacity to secrete protective molecules such as antimicrobial host defence molecules, cytokines and chemokines.

These results have been meanwhile confirmed at bronchial level in COPD patients, both in tissue and in broncho-epithelial cultures[12]. Our team showed a decrease of junctional proteins in COPD patients, while the number of vimentin cells was enhanced. We also showed an increase in the basal membrane height in these patients. These mesenchymal features are reactivated *in vitro*, and result at least in part from a reprogramming by TGF-β, as we showed that TGF-β promotes EMT in air-liquid interface broncho-epithelial cultures.

EMT is known to be induced either by environmental factors such as repeated injury and cigarette smoke[10] or extracellular mediators. Among them, TGF-ß1 is the key regulator of the remodeling pattern of CRS. TGF-β has been linked to COPD[13] and its expression is increased in the COPD airway epithelium[14]. However, its role in CRS is controversial. Higher levels of TGF-ß1 characterize CRSsNP, while CRSwNP patients show lower levels of TGF-ß1[15]. As EMT is present in both diseases despite their different expression of TGF-ß1, we speculate that other pathways may exist to induce EMT, and should thus be investigated in order to understand the regulatory mechanisms involved in epithelial plasticity and to provide specific treatments of these diseases.

EMT is observed in both CRSwNP and CRSsNP, but pIgR is only significantly reduced in CRSwNP. We also observed no correlation between pIgR expression and EMT markers (vimentin upregulation, E-cadherin and HmwCK downregulation), suggesting that the pathways involved in both mechanisms are probably different.

The strengths of this work rely on studies of well-characterized and carefully selected patients. Results are based on both nasal tissue and nasal secretions, and rely on protein and mRNA data. Previous studies in the field of upper/lower airway epithelium are reinforced and putative new mechanisms are suggested.

We recognize, however, some limitations of the present work, especially in the fact that these data should be confirmed in vitro, in differentiated air/liquid epithelial cultures. Despite some efforts and expertise of the lab with bronchial epithelial cultures, we did not achieve convincing results regarding pIgR production by cultured nasal epithelial cells, suggesting that terminal epithelial differentiation may not have been reached in these conditions.

Future directions of this work should thus include fully differentiated nasal epithelial cell culture, in order to address in vitro the effects of putative cytokines and eosinophils able to downregulate pIgR expression in chronic upper airway diseases. Regulation of pIgR expression and transcytosis of dimeric IgA across the epithelium (to generate secretory IgA at the apical pole) could be studied in this system, in response to factors that could directly affect pIgR expression; among these candidates are Th2 cytokines such as IL-4, IL-9, IL-13, and local eosinophils.

Another perspective could be to study the effects of therapies, especially methylprednisolone, on local IgA and pIgR expression, in prospective studies, as discussed in chapter 5, with correlation to clinical symptomatology.

Altogether, these two original studies shed new insights into the immunopathological pathways involved in CRS and might open possibilities for new therapeutic approaches in the treatment of these chronic upper airway diseases.

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